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Relationship between local changes in cortical blood flow and extracellular K^+ during spreading depression*

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Changes of local cerebral blood flow in response to a single cortical spreading depression of Lelko was studied in the brain. During spreading depression, potassium briefly accumulates in the brain extracellular space. The cortical blood flow was normal during the maximal rate of increase of the extracellular potassium concentration. The blood flow doubled during the subsequent period of normalization of potassium and remained high for one minute thereafter. Thus, potassium cannot be the immediate mediator of the blood flow increase.

Key words: Spreading depression, cerebral blood flow, brain extracellular potassium concentration, metabolic autoregulation of blood flow.

Lelko's cortical spreading depression (CSD) of EEG (Lelko 1944) is generalized response of the cerebral cortex of animals and man to a variety of noxious influences, chemical, electrical, and mechanical stimuli (Bures et al. 1974; Szabo et al. 1977).

The CSD is accompanied by localized changes of the extracellular concentrations of potassium, sodium chloride and calcium (Hansen 1979; Krug & Nicholson 1978; Nicholson & Krug 1975; Nicholson et al. 1977; Vyskočil et al. 1977). The perturbation moves from the site of elicitation at a speed of approximately 3 mm/min (Bures et al. 1974).

The response of the cerebral circulation to the passage of spreading depression was studied by Lelko (1944b), Buresova (1957), Tschilingi et al. (1957) and Van Harreveld & Ochs (1957), all of whom observed dilatation of pial vessels preceded by moderate constriction. From indirect evidence, Van Harreveld & Stamm (1952) deduced the presence of vascular constriction early in the course of the events that constitute CSD. The absolute magnitude of the local cerebral blood flow changes during CSD has never been reported, nor is the exact temporal relationship between ion concentration changes and cerebral blood flow known.

Since the changes of flow may be related to the extracellular potassium concentration ($[K^+]_e$) (Kuschinsky & Wahl 1978), we investigated the relationship between $[K^+]_e$ and local blood flow.

METHODS

Male Wistar rats, at average weighing 360 g, were anesthetized with an initial dose of pentobarbital (50 mg kg⁻¹) and supplementary doses as required later. Catheters were placed in the femoral arteries and one femoral vein. The spontaneously breathing rats were placed in a head holder and the parietal bones removed by craniotomy.

The experiment was performed as outlined in Fig. 1. Following respiratory steady-state, single CSD was elicited from the cortex with brief stab by hypodermic needle through small burr hole in the left frontal bone. The advance of the CSD was monitored with two double-barrelled potassium-sensitive microelectrodes with tip diameters of 1-2 μ m. The microelectrodes were passed through the intact dura and placed in the extracellular space of the left parietal cortex at depth of approximately

Presented in part at the meeting of the Scandinavian Physiological Society, Odense, Denmark, November 1978 (Quistorff et al. 1979) and the Symposium on Cerebral Metabolism and Neural Function, U.S. National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, Maryland, May 1979 (Gjedde et al., 1980b).

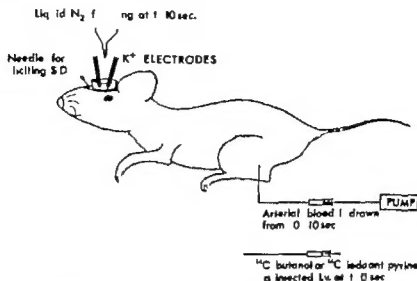


Fig. 1 Experimental design. A single cortical spreading depression (CSD) was elicited by a needle stab in the left frontal cortex. The advance of the CSD was followed by two potassium sensitive macroelectrodes placed in the left parietal cortex. Approximately 30 s after the initial increase of $[K^+]_o$ observed at the posterior electrode, regional cerebral blood flow was determined by bolus injection of either [14 C]butanol or [14 C]iodoantipyrine into a femoral vein and sampling of arterial blood. The left and right parietal cortices, covered by intact dural membrane, were frozen by application of liquid nitrogen after 10 s.

ly 0.5 mm (Hansen 1977). At the anterior-posterior distance between the electrodes was 3–4 mm, the CSD was travelled between the electrodes in one minute (the right parietal cortex was also exposed, but no electrodes were inserted).

In order to visualize the blood flow change accompanying the CSD, as well as to quantify the absolute blood flow rate, the tissue was freeze-clamped and the distribution of blood flow was measured by autoradiography and by liquid scintillation counting of small tissue samples.

Approximately 30 s after the initial change of $[K^+]_o$ detected at the posterior electrode, we injected a 200 μ l i.v. bolus of 250 μ Ci/kg 4-[N -methyl- 14 C]iodoantipyrine for autoradiography (1 rat) or 10 μ Ci/kg 0-[1- 14 C]butanol for tissue sampling (8 rats) and arterial blood sampling was started simultaneously. The arterial blood was sampled for 10 s at a known and constant rate by a syringe attached to a Harvard withdrawal pump. At the end of this blood collection, arrest of the cortical circulation *in situ* was produced by generous application of liquid nitrogen to the exposed dura overlying the parietal cortex. Since only a 0.2 mm wide cleft separates the dural surface from the arteries and veins situated at the surface of the cortex or in the subarachnoid space, freezing of the blood in the vessel supplying and draining the cortex, and thus arrest of the cortical circulation, can be estimated from the result of Quistorff (1980) who showed that the temperature of rat cortical tissue at a distance of 0.7 mm from a coolant with a temperature of less than -150°C will fall from 37°C to 0°C in 0.8 s. It may therefore be estimated that arrest of the cortical circulation was achieved about 1 s after application of the nitrogen and the termination of the collection of blood in the syringe. A

delay of a few seconds in the arrest of the cortical circulation would lead to a fall in $E(T)$ of below 10% for butanol and iodoantipyrine of a few per cent and thus to an underestimation of blood flow of similar magnitude. However, in that case the arterial sample collection would have been stopped before the moment of arrest of cortical circulation and the blood flow therefore overestimated, due to diversion of a too low value for $Q_b(T)$ in eq. (1). Thus, the net errors introduced by delayed freezing tend to cancel. The observed blood flow rates (see later) corresponded very closely to the blood flow rates observed by the same technique in similarly prepared, but decapitated rat (Sap & Duffy 1979).

A *autoradiography* for determination of local cerebral blood flow by labelled iodoantipyrine was performed as follows. The frozen brain was removed from the dural under liquid nitrogen and prepared for sectioning in a cryostat at -22°C . The parietal cortex was divided into 3 μ m tangential sections. Every fifth section was placed on a glass slide and rapidly dried on a hot plate (60°C). The dried sections were placed face down on the emulsion coated side of a Kodak Nuclear Medical Blue X-ray film for four days.

The *autoradiographic density* was measured using brain cortex section containing a known amount of isotope for calibration. Identical 0.5 mm brain sections were cut with a string knife (Franch et al. 1967) and smashed (about 20 mg) placed in 15 test tubes containing mock-CSF with addition of different amount of [14 C]iodoantipyrine. After 30 min the isotope was in equilibrium with the entire slice. Twenty μ m slices were then cut in a cryostat and some were rapidly dried for densitometry while others were weighed and counted by β -

Table 1 Physiological variables included in the present study

| variable | Mean \pm S.E. |
|-------------------------|-----------------|
| $\dot{V}O_2$ (ml/100g) | 95 \pm 3 |
| $\dot{V}CO_2$ (ml/100g) | 36 \pm 1 |
| Arterial pH | 7.41 \pm 0.02 |
| LABP* (mmHg) | 109 \pm 3 |

* Mean arterial blood pressure. Number of observations 10.

scintillation spectrometry. Films of standard and experimental sections were processed in Beckman densitometer with beam aperture of 0.2 mm².

Tissue sampling for determination of regional cerebral blood flow by labelled botanotol was performed as follows. The frozen brain was divided into 1.2 mm thick coronal slices by an electric saw in glass box at -25°C (Quistorff 1971). The 5 mm wide bits of cortical tissue was isolated quickly and prepared for liquid scintillation counting, as in the arterial samples.

The blood flow of the cortical regions was calculated as described previously (Gjedde et al. 1980) from tissue and arterial blood sample contents of labelled botanotol or labelled radioisotopyrine using an equation relating the integrated in- and efflux rates of botanotol in brain

$$F^* = \frac{1}{E(T)} F^* = \frac{C_{in}(T)}{C_{out}(T)} \quad (1)$$

in which $E(T)$ is the net cerebral extraction fraction of radioactive tracer in time T after the introduction of the indicator into the circulation, F^* the constant rate of collection of arterial blood, $C_{in}(T)$ the amount of radioactive tracer collected, and $C_{out}(T)$ the amount of indicator per unit weight of sample of brain to which F^* refers.

The use of eq. (1) requires that two criteria are fulfilled: (1) that the time integrals of arterial and femoral arterial concentrations of tracer from injection to arrest of cortical circulation are equal, and (2) that efflux of tracer from brain is negligible in the 10 s and that therefore

$E(10\text{ s}) \approx 1$ may be used as good approximation.

With regard to the first criterion, Gjedde et al. (1980a) showed that the integral was independent of the site of sampling from 5 to 30 s after injection of the tracer.

The second criterion was confirmed by Gjedde et al. (1980a) for botanotol and by Sakurada et al. (1978) for radioisotopyrine by comparison of cerebral blood flow values calculated with these indicators with values measured directly on the basis of arterial and venous concentrations or by means of inert gases of known, essentially infinite, permeability in the blood-brain barrier.

In addition, Crome (1945) showed that the extraction fraction of botanotol, integrated from the time of appearance of an autoradiodark bands in cerebral venous blood to time later averaged 0.96 in the dog.

The criteria are confirmed in rat in which the net extraction fraction of botanotol at 10 s was measured directly by simultaneous sampling of blood from both

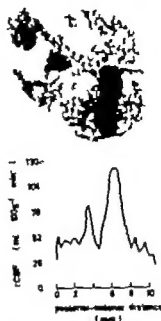


Fig. 2 Upper panel: Autoradiogram of tangential (horizontal) 20 μm section of rat brain parietal cortex, cut at depth of 0.5 mm. A single spreading depression was elicited in the left hemisphere. The cortex was frozen in situ by application of liquid nitrogen, ten seconds after an i.v. bolus injection of labelled radioisotopyrine. The frozen cortex was cut in cryostat and the sections placed on an X-ray film for 4 days. The lower part of the autoradiogram represents the left parietal cortex. The spreading depression moved from the right (anterior) part of the section to the left (posterior) one. For $E(T) \approx 1$ the radioactivity of the sample, i.e. the grain density is linearly proportional to the local blood flow as shown in eq. (1).

Lower panel: Densitometric scan through the left hemisphere of the autoradiogram shown in the upper panel. Densities were converted to blood flow rates by the procedure described in the text.

femoral arteries, one external carotid artery and the superior sagittal sinus of the brain. The amounts of tracer recovered in the superior sagittal sinus and external carotid samples in the 10 s from injection represented 6.5% and 96.3% respectively of the average amount collected in the femoral samples.

RESULTS

The physiological variables of the animals are given in Table 1. The autoradiographs (Fig. 2, upper panel) revealed a remarkable picture: An approximately 2 mm wide well-defined band of increased density on the hemisphere where the CSD wave progressed. The wide band of increased density

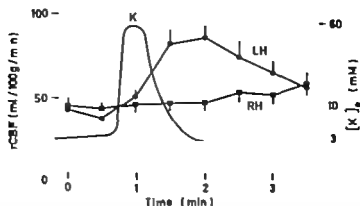


Fig. 3. Changes of extracellular potassium concentration and regional cerebral blood flow (rCBF) of 3 mg samples of rat brain parietal cortex during a single cortical spreading depression in the left hemisphere. The cortex was frozen *in situ* by application of liquid nitrogen 10 s after an intravenous bolus injection of radioactive butanol. The frozen brain was cut in 1.2 mm thin coronal sections and prepared for liquid scintillation spectrometry. The local blood flow was calculated from the radioactivity of each sample. The progression of the parietal spreading depression was monitored by potassium-sensitive microelectrodes placed in the cortex of the left hemisphere. From the width of the cortical slices and assumption of uniform speed of progression corresponding to that observed between the electrodes, spatial flow events were converted to temporal events at the site of one electrode. The trace marked K shows the changes of extracellular potassium concentration at one electrode in the left parietal cortex. The curve marked LH represents the calculated change of blood flow at the same electrode. The curve marked RH represents the change of blood flow at a corresponding site in the right parietal cortex. The bars represent one standard error of the mean.

was preceded by a similarly dense but narrow band. These areas were separated by a somewhat wider band of normal density. The zones of increased density transected the entire cortex.

Close examination of the autoradiographs revealed a dotted pattern of increased density in the unaffected cortical regions of the left hemisphere as well as in the cortical regions of the right hemisphere (Fig. 2 upper panel). The dots measured approximately 0.4 mm in diameter and probably reflect the presence of cortical columns (Hubel & Wiesel 1962; Mountcastle 1957; Sokoloff et al. 1977).

The result of the quantitation procedure is shown in the lower panel of Fig. 2. Calculated values for cortical blood flow in the undisturbed parietal cortex varied between 50 and 40 ml/100 g min⁻¹; the variations corresponding to areas of different densities. The scanner aperture of 0.2 mm led to underestimation of the differences in flow in areas with steep density gradients.

The local blood flow determined by labelled butanol contents in tissue samples confirmed the picture obtained from the autoradiographs (Fig. 3). However, the initial narrow band of increased

blood flow could not be detected, probably because the lengthwise resolution power of the tissue sampling methods was lower than that of the autoradiogram scanning method (approximately 1.2 mm versus 0.5 mm).

The observation that the flow of the undisturbed cortex was 50% of the flow normally observed in rat cortex reflected the barbiturate anaesthesia (Gjedde et al. 1980b; Nilsson & Siesjö 1975). Close scrutiny of the relationship between the $[K^+]_o$ and cortical blood flow revealed the presence of three phases. Initially, the $[K^+]_o$ increased to 60 mM while the cortical blood flow remained unchanged. During the second phase, cortical blood flow increased to twice the normal value while $[K^+]_o$ turned to 3 mM. During the final phase, which lasted about one minute, cortical blood flow decreased to its previous level and no further change of $[K^+]_o$ was noticed.

DISCUSSION

The present study demonstrates for the first time the marked change in cerebral blood flow occurring during a single CSD in the rat. The CSD represent

phenomenon in which significant variations of K^+ and blood flow occur in the cerebral cortex. Therefore the phenomenon is ideally suited for a study of the relationship between the two variables. A prerequisite for this study was the development of a method which can detect rapid flow changes (Gjedde *et al.* 1980).

It is evident from the present study that high cerebral blood flow was not directly associated with the increased $[K^+]_i$ but rather with the subsequent reduction of $[K^+]_i$. It is well known that increased cerebral blood flow is coupled to increased metabolic activity in the brain (Renshaw *et al.* 1975) and that high $[K^+]_i$ stimulates the metabolic rate of nervous tissue (Ashford & Dixon 1935; Hertz & Clausen 1963; Lothman *et al.* 1975; Shimohara *et al.* 1979). Thus, it is likely that the effect of increased $[K^+]_i$ on cerebral blood flow is mediated through metabolic factors that may be related to increased ion pumping in connection with the return of potassium to the intracellular compartment (Quistorff *et al.* 1979). The metabolic factor(s) that constitute(s) the link between metabolites and flow remain(s) to be identified.

In the past, several factors including O_2 , H^+ , K^+ and adenosine have been implicated in metabolic autoregulation. However it has not been possible to demonstrate parallel changes of any of these factors in all the conditions of increased blood flow investigated (Astrup *et al.* 1976; Leniger-Follert & Lübbers 1979; Rubio *et al.* 1975).

The flow variations during a CSD can partially be explained by an action of potassium itself on cerebral vessels. It has been shown that an increase of $[K^+]_i$ up to 10 mM caused dilatation of pial vessel while further increases lead to constriction (Kutschinsky & Wahl 1978). Thus a marked transient increase of $[K^+]_i$ would initially induce dilatation of cerebral vessels, followed by a vasoconstriction subsequently cellular re-uptake of K^+ (and extrusion of Na^+ (Hansen 1979)) could give rise to metabolically-induced vasodilatation. This succession of events was actually observed in the autoradiographs obtained in this study. Thus, the first peak of increased density corresponds to the initial increase of $[K^+]_i$, followed by vasoconstriction (the intermediate band of decreased density) ending in a marked longer lasting vasodilatation, during which K^+ is pumped back into the cells.

The present data suggest that the major perturbation of cerebral blood flow occurs after the wave of

$[K^+]_i$ increase. The cellular events immediately preceding the changes of extracellular ion concentrations during CSD are still unknown.

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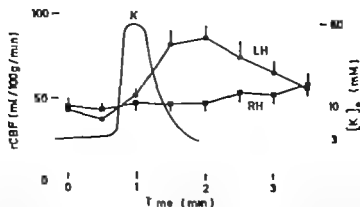


Fig 3 Changes of extracellular potassium concentration and regional cerebral blood flow (rCBF) of 3 mg samples of rat brain parietal cortex during a single cortical spreading depression in the left hemisphere. The cortex was frozen *in situ* by application of liquid nitrogen 10 s after an intravenous bolus injection of radioactive butanol. The frozen brain was cut in 1 mm thin coronal sections and prepared for liquid scintillation spectrometry. The local blood flow was calculated from the radioactivity of each sample. The progression of the parietal spreading depression was monitored by potassium-sensitive microelectrodes placed in the cortex of the left hemisphere. From the width of the cortical slices, and assumption of uniform speed of progression corresponding to that observed between the electrodes, spatial flow events were converted to temporal events at the site of one electrode. The trace marked K shows the changes of extracellular potassium concentration at one electrode in the left parietal cortex. The curve marked LH represents the calculated change of blood flow at the same electrode. The curve marked RH represents the change of blood flow at a corresponding site in the right parietal cortex. The bars represent one standard error of the mean.

was preceded by a similarly dense but narrow band. These areas were separated by a somewhat wider band of normal density. The zones of increased density transected the entire cortex.

Close examination of the autoradiographs revealed a dotted pattern of increased density in the unaffected cortical regions of the left hemisphere as well as in the cortical regions of the right hemisphere (Fig 2 upper panel). The dots measured approximately 0.4 mm in diameter and probably reflect the presence of cortical columns (Hubel & Wiesel 1962; Mountcastle 1957; Sokoloff et al 1977).

The result of the quantitation procedure is shown in the lower panel of Fig 2. Calculated values for cortical blood flow in the undisturbed parietal cortex varied between 50 and 40 ml (100 g)⁻¹ min⁻¹; the variations corresponding to areas of different densities. The scanner aperture of 11 mm² led to underestimation of the differences in flow in areas with steep density gradients.

The local blood flow determined by labelled butanol contents in tissue samples confirmed the picture obtained from the autoradiographs (Fig 3). However, the initial narrow band of increased

blood flow could not be detected, probably because the lengthwise resolution power of the tissue sampling methods was lower than that of the autoradiogram scanning method (approximately 1 mm versus 0.5 mm).

The observation that the flow of the undisturbed cortex was 50% of the flow normally observed in rat cortex reflected the barbiturate anaesthesia (Gjedde et al 1980b; Nilsson & Siejō 1975). Close scrutiny of the relationship between the $[K]_o$ and cortical blood flow revealed the presence of three phases. Initially, the $[K]_o$ increased to 10 mM while the cortical blood flow remained unchanged. During the second phase, cortical blood flow increased to twice the normal value while $[K]_o$ returned to 3 mM. During the final phase, which lasted about one minute, cortical blood flow decreased to its previous level and no further change of $[K]_o$ was noticed.

DISCUSSION

The present study demonstrates for the first time the marked change in cerebral blood flow occurring during a single CSD in the rat. The CSD represents

Oxygen supply, oxygen consumption and endocrine and exocrine secretions of the isolated, perfused porcine pancreas

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HOLST J. J., LINDKJÆR JENSEN S., NIELSEN O. V. & SCHWARTZ, T. W. Oxygen supply, oxygen consumption and endocrine and exocrine secretions of the isolated, perfused, porcine pancreas. *Acta Physiol Scand* 1980 109: 7-13. Received 14 July 1979. ISSN 0001-6772. Institute of Medical Physiology C, University of Copenhagen, Department of Surgery C, Rigshospitalet, Copenhagen and the Institute of Medical Biochemistry, Aarhus University, Denmark.

We studied the effect of varying oxygen supply on the endocrine and exocrine secretions of the isolated perfused porcine pancreas utilizing completely synthetic perfusion medium with and without the addition of erythrocytes. With synthetic medium oxygenated to a P_{O_2} of 300 mmHg, oxygen consumption was constant for flow rates at or above 0.5 ml min⁻¹ g⁻¹ (et weight). Addition of erythrocytes to the medium did not increase oxygen consumption at flow rates above this level. Furthermore, the secretion of fluid, bicarbonate and protein in response to secretin and acetylcholine was not influenced by addition of erythrocytes. Similarly, the secretion of insulin, glucagon, pancreatic polypeptide, and somatostatin in response to arginine and acetylcholine was unchanged. Arginine stimulated the secretion of all four peptides, whereas acetylcholine stimulated the secretion of insulin and pancreatic polypeptide and inhibited glucagon and somatostatin secretion. The results indicate that the porcine pancreas is respiring adequately when perfused with completely synthetic perfusion medium at flow above about 0.5 ml min⁻¹ g⁻¹ and P_{O_2} about 300 mmHg, and that addition of erythrocytes is not necessary for the study of its secretory functions.

The isolated perfused pancreas has been used extensively in studies of pancreatic endocrine and exocrine secretion (Ross 1972; Scratcherd & Case 1973). In our studies of the secretory effects of gastrointestinal hormones and transmitter substances on the pancreas (Lindkær Jensen et al. 1978a, b; 1979; Schwartz et al. 1978) we found it essential to make use of the isolated perfused pancreas, in order to avoid neural or endocrine effects other than those under study. The porcine pancreas was chosen because the majority of the gastrointestinal hormones are known in their porcine form only. The oxygen supply to the isolated perfused organ preparation is usually provided either by oxygenation of synthetic perfusion medium to a

high partial pressure of oxygen, or by the addition of erythrocytes to the medium before oxygenation. However, a comparison between the two methods, in which endocrine as well as exocrine secretions are considered, has not been carried out.

In the present report such a study is described.

METHODS AND MATERIALS

The pancreatic glands from 13 young pigs of Danish Landrace (approx. 25 kg) were studied. Isolation and perfusion was performed as previously described (Lindkær Jensen et al. 1978; Lindkær Jensen et al. 1978). In short, the glands were isolated together with 12 cm segment of the aorta, comprising the celiac trunk as well as the superior mesenteric artery. The duodenum was not included in the

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ble. Oxygen consumption ($\mu\text{l O}_2 \times \text{g} \times \text{min}^{-1}$) at various flow rates with or without addition of erythrocytes to the perfusion medium.

Each of 6 perfusions, mean values based on 1-4 duplicate determinations were calculated and these data are here summarized as mean \pm S.E. of the 6 perfusions.

| | Flow rate ($\text{ml} \times \text{g}^{-1} \times \text{min}^{-1}$) | | |
|----------------------|---|-----------------|-----------------|
| | 0.30 \pm 0.01 | 0.48 \pm 0.02 | 0.72 \pm 0.03 |
| without erythrocytes | 3.18 \pm 0.19 | 6.31 \pm 0.51 | 7.05 \pm 0.36 |
| with erythrocytes | 7.99 \pm 0.43 | 8.84 \pm 0.52 | 7.47 \pm 0.5 |

the basal level of secretion again. The medium was then supplemented with erythrocytes and the entire protocol was repeated except that the flow changes were made after the incubation periods. In 3 experiments the gland was perfused with erythrocyte-containing medium first and synthetic medium later.

11 glands were perfused with synthetic medium only. Flow rates ranging from 0.37 to 0.63 $\text{ml} \times \text{min}^{-1} \times \text{g}^{-1}$ for 40 min and oxygen consumption determined every 30 min.

with vs.

concentrations in pancreatic juice of protein and bicarbonate and in effluent of amylase, glucose and pancreatic polypeptide were measured as previously described (Andersen, Jensen et al. 1978a; Schwartz et al. 1978). Somatostatin concentrations in effluent were measured by radioimmunoassay. The antiserum, R 213 was raised in a rabbit against synthetic somatostatin consisting of bovine and human albumin (Reisfeld et al., unpublished data). Standards were synthetic, cyclic somatostatin (Boehringer Bioproducts, Geneva, Switzerland), and the assay in ^{125}I -tyr-somatostatin labelled according to Lundquist (1973) (a generous gift from NOVO Research Institute, Bagsvaerd, Denmark). Detection limit of the assay was 1 pmol/l, interassay coefficient of variation better than 6% in the working range. Bound and free somatostatin were separated with plasma-coated charcoal. The antiserum reacts equally well with reduced and cyclic somatostatin and also binds equally well ^{125}I -somatostatin with tyrosine substituted in position 1 and position 11. The antiserum does not crossreact with other known pancreatic peptides. The results of recovery studies performed on the effluent from the perfused pancreas deviated less than 10% from the expected values.

Calculation

The hormone outputs were calculated on the basis of concentration determinations in all effluent samples. The total output during stimulation was calculated as the sum of the 1-min outputs. The hormone outputs in the immediately preceding 10 or 5 min periods were calculated in the same way for comparison (Fig. 3). In Fig. 3 the data are presented as (average) outputs per min. Statistical evaluation as performed by means of Student's *t*-test for paired data. The level of statistical significance was chosen at 0.05.

RESULTS

Influent and effluent values of P_{O_2} , pH and P for the 6 glands which were perfused with and without erythrocytes appear in Table 1 and oxygen consumption values calculated on the basis of a coefficient of solubility for oxygen in perfusion medium of 0.023 and oxygen binding capacity of hemoglobin of 1.39 $\text{ml} \times \text{g}^{-1}$ are shown in Table 2. Influent P values were slightly lower with erythrocytes than without and ranged between 400 and 500 mmHg. Without erythrocytes oxygen consumption was significantly lower at a flow rate of 0.3 $\text{ml} \times \text{min}^{-1} \times \text{g}^{-1}$ than at 0.48 $\text{ml} \times \text{min}^{-1} \times \text{g}^{-1}$ but was not increased by raising the flow rate to 0.72 $\text{ml} \times \text{min}^{-1} \times \text{g}^{-1}$. In agreement with this observation, the effluent P_{O_2} increased from 60 ± 3 to 204 ± 18 mmHg by the latter increase in flow rate. To substantiate further that the maximum oxygen consumption was reached at a perfusion rate of approximately 0.5 $\text{ml} \times \text{min}^{-1} \times \text{g}^{-1}$ we determined

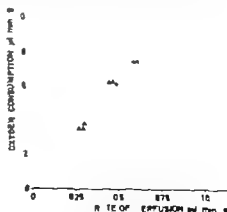


Fig. 1 The relationship between oxygen consumption and rate of perfusion without erythrocyte addition. The filled circles (●) represent the mean of 6 determinations in each of seven perfusion experiments. The data indicated by ○ are the individual mean values from Table 2.

Table 1 P_{O_2} , pH and P_{CO_2} values from the isolated perfused porcine pancreas

Influent (A) and effluent (V) P_{O_2} , pH and P_{CO_2} from perfusion of 6 isolated pancreatic glands. For each pancreas values based on 1-4 duplicate determinations were calculated and these data are summarized as mean followed by S.E. of the 6 perfusions.

| Flow (ml \times min \times g ⁻¹) | Sample | P without erythrocytes | P with erythrocytes | pH without erythrocytes | pH with erythrocytes | P_{CO_2} without erythrocytes | P_{CO_2} with erythrocytes |
|--|--------|---------------------------|------------------------|----------------------------|-------------------------|------------------------------------|---------------------------------|
| 0.30 \pm 0.01 | A | 440 \pm 12 | 397 \pm 59 | 7.45 \pm 0.01 | 7.36 \pm 0.02 | 36.4 \pm 1 | 43 \pm 3 |
| | V | 48 \pm 7 | 3 \pm 9 | 7.18 \pm 0.02 | 7.21 \pm 0.03 | 61 \pm 6 | 58 \pm 3.8 |
| 0.48 \pm 0.02 | A | 488 \pm 5 | 424 \pm 40 | 7.41 \pm 0.02 | 7.39 \pm 0.04 | 34.7 \pm 0 | 38.8 \pm 3.8 |
| | V | 60 \pm 3 | 41 \pm 5 | 7.35 \pm 0.02 | 7.35 \pm 0.04 | 50.9 \pm 2.4 | 53.4 \pm 4.4 |
| 0.72 \pm 0.03 | A | 514 \pm 12 | 438 \pm 68 | 7.44 \pm 0.01 | 7.4 \pm 0.07 | 41.8 \pm 4.6 | 39.9 \pm 6 |
| | V | 704 \pm 18 | 182 \pm 45 | 7.25 \pm 0.04 | 7.21 \pm 0.01 | 54.8 \pm 3.8 | 54.3 \pm 4 |

preparation but the pancreaticoduodenal vascular supply to the pancreas remained intact. Thus the pancreas was perfused through its entire arterial supply i.e. the splenic, the gastroduodenal and the superior mesenteric arteries, and drained through its entire contributions to the portal vein.

The pancreatic gland were perfused at 38°C with Krebs-Ringer bicarbonate solution supplemented with fumarate, glutamate and pyruvate each 5 mmol/l. Dextran T 70 (Pharmacia Fine Chemicals, Uppsala, Sweden) 40 g/l, human serum albumin (Trocken reinst Behringwerke GFR) 1 g/l and glucose 3.5 mmol/l. The perfusate was oxygenated with 96% oxygen, 4% CO using a multiple bulb oxygenator (Ross 1977b) and the flow was controlled by an adjustable finger pump (Ole Dich, Copenhagen). Perfusion pressure did not exceed 90 mmHg. The effluent was collected for 1 min periods by means of an automatic fraction collector centrifuged (when erythrocytes were present) and frozen immediately at -70°C. The pancreatic duct was cannulated with a polyethylene catheter (dead space 150 μ l) and pancreatic secretions collected for 5 or 15 min periods and frozen.

Influent and effluent samples for gas analysis were obtained close to the pancreas every 10-30 min in gas tight syringes under anaerobic conditions and analysed for P_{O_2} , P_{CO_2} , pH, HCO_3^- , hemoglobin concentration and saturation of oxygen using the Acid Base Laboratory II Radiometer Copenhagen Denmark and standard oxygen electrodes (Radiometer Copenhagen). Oxygen consumption was calculated as flow \times the arteriovenous concentration deficit of physically dissolved and chemically bound oxygen.

Pancreatic exocrine secretion was stimulated by addition of secretin (pure porcine secretin, GH Research Laboratories, Stockholm, Sweden) to the influent for 5 min (by means of a side-arm pump) to a final concentration of 300 pmol/l or by acetylcholine (also 5 min) (Sigma Chemical Company, Miss USA) 10⁻⁶ mol/l. Endocrine secretions were stimulated by addition of arginine 5 mmol/l for 10 min.

Erythrocytes were prepared from outdated human bank blood stored for 3 weeks in Citrate Phosphate Dextrose. The erythrocytes were washed several times with saline and perfusion buffer filtered and added to the perfusion

medium reservoir to a hematocrit of 10%. pH was necessary—adjusted by means of NaHCO₃ 0.1 mol/l.

Experimental protocol

1-6 glands. After 30 min perfusion (equilibrium period) about 0.5 ml/g \times min with medium without erythrocytes the flow rate was adjusted to 0.48 \pm 0.02 ml/g \times min \pm 0.01 ml/g \times min, 0.72 \pm 0.03 ml/g \times min and again 0.5 ml/g \times min each period lasting 10 min.

The glands were then stimulated with the following agents at a flow of (approximately) 0.5 ml/g \times min: arginine 10 min followed by (at least) 15 min rest, acetylcholine 5 min and 10 min rest, secretin for 5 min and 10 min rest. We knew from previous experience that the rest periods were of sufficient length for the glands.

The ABL-II (Radiometer Copenhagen, Denmark) is an automatic bloodgas analyser which measures pH, P_{O_2} , P_{CO_2} and hemoglobin concentrations and calculates bicarbonate, standard bicarbonate, total CO₂ and—by solving a Hill-equation with a Bohr factor of -0.48 and a P_{50} of 26.6 mmHg—calculates oxygen saturation. However, the machine cannot reliably measure the P_{O_2} of solutions which do not contain hemoglobin. Therefore determinations of P_{O_2} in medium without erythrocytes were performed with a standard oxygen electrode as well. Furthermore, by comparing the hemoglobin determinations of the ABL-II with results from conventional method on identical samples of erythrocyte-containing perfusate it was found that the former exceeded the latter by a factor 1.31. Hemoglobin values were corrected accordingly. Finally, the P_{50} for the hemoglobin binding of oxygen used in the calculations 26.6 mmHg is probably higher than the P_{50} of erythrocytes stored for 3 weeks in CPD which has been reported to be approximately 20 mmHg (Husman et al. 1969) therefore all oxygen saturations were calculated using the following equation:

$$S = 100 \times \frac{P_{O_2}^n}{(20.0)^n + P_{O_2}^n} \quad \text{where}$$

$$Z = P_{O_2} \times 10^{-4} = \text{mmHg}$$

This equation is identical to the one used by the ABL-II apart from the substitution of 26.6 with 20.

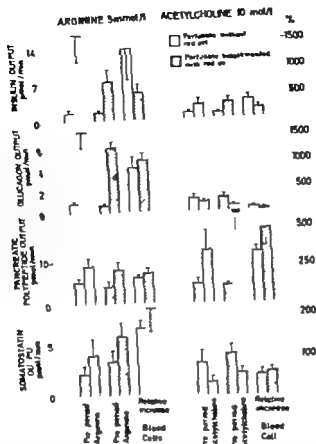


Fig. 3. Endocrine secretion of the isolated porcine pancreas, perfused with or without erythrocytes. The columns represent means \pm S.E. ($n=6$) of hormone outputs before and during stimulation. In addition, the relative response to stimulation—given as output during stimulation in percentage of basal output—is shown in the right hand columns.

gen consumption of an isolated pancreas perfused with a synthetic medium. The only way to define whether the oxygen requirements are met *in vitro* is to study the relationship between flow rate and oxygen consumption: the flow rate above which the oxygen consumption cannot be increased, they should then provide sufficient oxygen for the preparation. A flow of this limiting magnitude leaves no room for full coverage of oxygen requirements during stimulation, and to increase either the oxygen supply by larger increases in flow rate may apart from being prodigal of perfusion medium, be unexpedient, since at high flow rates with synthetic medium there is a risk of edema formation. Furthermore, with the pancreas, the fact that the majority of the tissue—the exocrine gland,

respires adequately does not provide a guarantee that the endocrine gland is also adequately perfused.

To solve these problems we studied the exocrine and endocrine secretions of the isolated porcine pancreas in response to various stimuli during perfusion with and without erythrocytes: by addition of erythrocytes the oxygen supply could be increased without increasing the flow rate. Our results show that at or above a flow rate of $0.5 \text{ ml} \times \text{min}^{-1} \times \text{g}^{-1}$ and with an influent oxygen tension of about 500 mmHg, the oxygen consumption of the pancreas could not be increased further (in the basal state) by increasing flow rate and the chosen flow rate should therefore ensure adequate oxygenation under such conditions. Furthermore, addition of

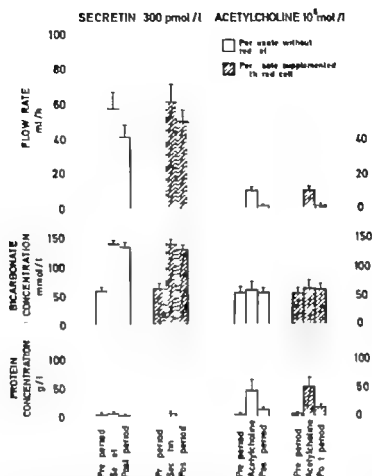


Fig 2 Exocrine secretion of the isolated pancreas perfused with or without erythrocytes. The columns represent mean \pm S.E. ($n=6$) of flow of juice bicarbonate and protein concentrations before during and after stimulation

the oxygen consumption of further seven glands perfused at flow rates ranging from 0.37 to 0.62 ml \times min \times g. The combined results are shown in Fig 1. There is a nearly linear relationship between oxygen consumption and flow rate up to approximately 0.5 ml \times min \times g. Above this flow rate oxygen consumption appears to remain constant. During perfusion with erythrocytes oxygen consumption remained constant over the flow range tested (Table 2) and identical to that obtained during perfusion at 0.72 ml \times min \times g. at 0.48 ml \times min \times min oxygen consumption was 40% higher with than without erythrocytes.

The basal exocrine secretion was uninfluenced by erythrocyte addition. The exocrine responses to secretin and acetylcholine appear in Fig 2. secretin stimulated flow and bicarbonate secretion and the results are comparable to previously reported results (Lindkær Jensen et al 1978a). Acetylcholine stimulated flow and protein secretion significantly.

Virtually identical results were obtained with or without the addition of erythrocytes.

The endocrine secretions appear in Fig. 3. 4. Somatostatin significantly stimulated the secretion of pancreatic hormones whether erythrocytes were present or not and the stimulated outputs relative to the basal outputs were not significantly different. Acetylcholine significantly stimulated insulin and pancreatic polypeptide secretion and significantly inhibited glucagon and somatostatin secretion. Similar results were found after addition of erythrocytes. The concentration variations in a single typical experiment are shown in Fig. 4.

DISCUSSION

Because of the complexity of the venous drainage of the pancreas it is difficult to measure its oxygen consumption in vivo. The investigator is therefore left with some uncertainty when evaluating the

acetylcholine on somatostatin secretion has not been reported before: lower concentrations of acetylcholine however have no effect and the dose to 10^{-6} mol/l is probably not a biological action since this dosage (a) is far below the D_{50} for e.g. stimulation of pancreatic peptide secretion (Schwartz et al. 1978) and (b) is to an increase of the vascular resistance of crests (unpublished studies). The effects of acetylcholine on the exocrine secretion of the isolated porcine pancreas have not been studied before and the results are of great interest in view of marked effects of vagal stimulation on the pancreatic exocrine secretion in the pig (Hickson 1970; Iat et al. 1978). In contrast to the effect of ACh stimulation, however acetylcholine does not affect the concentration of bicarbonate in the secreted by the isolated perfused pancreas. These findings are in agreement with the hypothesis, that the vagally induced bicarbonate reflow is not entirely mediated by cholinergic transmission (Holst et al. 1978; Fahrenkrug et al. 1979). The present results support the conclusion, put forward in our previous reports (Lindkær Jensen 1978; b) that the isolated porcine pancreas, perfused with a completely synthetic medium at P. of 10 mmHg is a reliable tool in the study of pancreatic endocrine and exocrine physiology.

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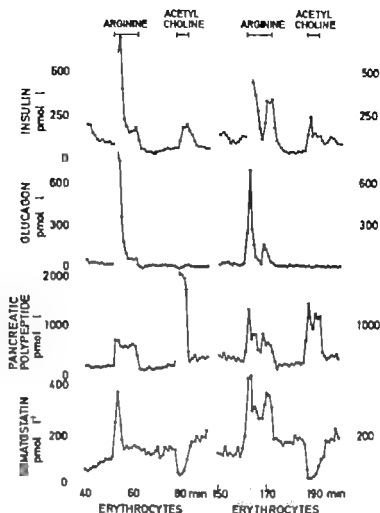


Fig. 4 Hormone concentrations in the effluent from an isolated perfused pancreas in response to arginine and acetylcholine with and without erythrocytes in the perfusate. Results of a single typical experiment.

erythrocytes during the higher flow rate—still in the basal state—did not increase oxygen consumption further.

The oxygen consumption during perfusion at a mean flow of $0.48 \text{ ml} \times \text{min}^{-1} \times \text{g}^{-1}$ with erythrocytes did not differ from those obtained at the higher and lower flow rates but differed slightly and significantly from that obtained without erythrocytes, indicating that the flow rates below the average value of approximately $0.5 \text{ ml} \times \text{min}^{-1} \times \text{g}^{-1}$ were insufficient to ensure complete oxygenation. Nevertheless the endocrine and exocrine secretions basal as well as stimulated were comparable whether erythrocytes were added or not. Therefore perfusion with a completely synthetic medium without erythrocytes at a flow which must be maintained at at least $0.5 \text{ ml} \times \text{min}^{-1} \times \text{g}^{-1}$ (or somewhat above) and $\approx 300 \text{ mmHg}$ appears adequate

for the study of the endocrine and exocrine secretions of the porcine pancreas.

Some of the secretory results deserve comment. The effect of acetylcholine on insulin secretion is in agreement with previous reports (Iversen 1973; Loubatières-Mariani et al. 1973) on cholinergic effects on rat and dog pancreas. In the present experiments however acetylcholine did not stimulate glucagon secretion (on the contrary a significant inhibition was noted) in agreement with our previous observations in pigs (Holst et al. 1977). In dogs acetylcholine stimulates glucagon secretion (Iversen 1977; Kaneto & Kosaka 1975) also in our experiments. Undoubtedly we are facing important species differences (Larsson et al. 1978). An inhibitory effect

Depression of mechanical performance by active shortening during twitch and tetanus of vertebrate muscle fibres

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EDMAN A. P. Depression of mechanical performance by active shortening during twitch and tetanus of vertebrate muscle fibres. *Acta Physiol Scand* 1980, 109: 15-26. Received 19 July 1979. ISSN 0001-6772. Department of Pharmacology, University of Lund, Sweden.

Shortening during activity of frog single muscle fibres caused graded depression of the contractile force that persisted for 800-900 ms during a partially fused or completely fused tetanus. The depression of force was not associated with change of the shortening velocity at zero load. Passive shortenings performed just before stimulation had no effect on the subsequent course of contraction. The decrease in isometric force produced by shortening was not significantly affected by a twitch applied immediately before or after the shortening phase. For a given amount of shortening the depressant effect during a fused tetanus was 8-28% of that produced during a twitch. The effect was substantially reduced, both during twitch and tetanus, in the presence of 0.5 mM caffeine. The length dependence of the movement effect was studied between 1.7 and 2.9 μ m sarcomere spacing. Maximum depression of force (in per cent of the control at each length) was obtained at 2.1-2.3 μ m sarcomere length, the effect being steadily reduced at shorter and more extended lengths. The Q_{10} of the depressant effect was 2.85 ± 0.16 (S.D.). The features of the movement effect are consistent with a true desorption of the contractile system as would occur if shortening reduced the binding of activator calcium to the regulatory proteins of the myofilaments.

Key words: Skeletal muscle fibres, force depression by shortening, shortening induced deactivation.

vertebrate skeletal muscle which is allowed to shorten during a twitch loses some of its contractile strength during the remainder of the activity period (Weill & Wilkie 1960; Hill 1964; Joyce & Rack 1969; Joyce, Rack & Westbury 1969; Edman & Westling 1971; Briden & Alpert 1977; Dickinson & Oldidge 1977). A more detailed study of this phenomenon during twitch and partially fused tetanus has previously been performed on isolated frog muscle fibres (Edman 1975). It was shown that the depressant effect is long-lasting and does not appear merely by restimulation of the fibre. It is furthermore demonstrated that the effect is significantly dependent on the load, or the work output during the shortening phase. These findings argue against the idea that the reduced tension development after the movement is due to enhanced re-uptake of activator calcium by the sarcoplasmic reticulum or to exhaustion of some energy substrate for the contractile process (cf. Hill 1964). It was suggested, as a more plausible

explanation of the movement effect (Edman 1975) that shortening causes a change in the myofilament system itself that leads to a reduced degree of interaction between the A and I filaments.

The present investigation extends the previous study of the movement effect to include recordings also during a fused tetanus. Experiments have been designed to investigate if the depressant effect is dependent on the area of overlap between the A and I filaments, the temperature and the degree of activation of the contractile system when the movement occurs. Attempts have also been made to find out if the decrease in isometric force is associated with a similar change of the shortening velocity at zero load. Some of these results have been reported in a preliminary form (Edman 1976).

METHODS

Preparation. Single fibres from the semitendinosus and tibialis anterior muscles of *Rana temporaria* were studied. The essentials of the techniques used for dissection and

Table 1. Comparison between depressant effect of active shortening during single twitch partially fused and completely fused tetanus

| | Amplitude of shortening in excess of control ($\mu\text{m}/\text{sarcomere}$) | Depression of peak force (% control) | |
|------|---|--------------------------------------|---------------|
| | | Single twitch | Fused tetanus |
| 1975 | 0.46 | | 40.5 |
| 1976 | 0.16 | 29.7 | 7.5 |
| 1976 | 0.20 | 47.5 | 4.0 |
| 1977 | 0.14 | | 5.2 |
| 1979 | 0.19 | 48.4 | 7.0 |
| 1979 | 0.16 | 51.3 | 4.6 |
| | | | 4.0 |

length was adjusted to 2.25 μm . The measurements of the sarcofascicular area was performed in a separate trough at 100 \times magnification as previously described (Edman 1975).

Recording and measurement of responses. Tension and length changes were displayed on Tektronix 5103M storage oscilloscope and photographed on 35 mm film. Measurements from the film records were made in a Kon model 6C profile projector at 10 \times magnification during 1979.

Velocity of calculated shortening. The velocity of shortening at zero load, V , was measured using the multiple flash method described previously (Edman 1979). As constructed in the previous paper V is constant between 1.65 and approximately 2.7 $\mu\text{m}/\text{sarcomere}$ length. All measurements in the present study were performed between 1.80 and 2.0 μm sarcomere spacing as exemplified in Fig. 7.

RESULTS

Comparison of force depression by shortening during twitch and tetanus

The approach used for determination of the depressant effect of active shortening was similar to that previously described (Edman 1975). The contractile force was relaxed at approximately 2.4 μm sarcomere length and the fiber was released during contraction to shorten and redevelop force at a new length between 2.25 and 1.95 μm , i.e. within a range where the tetanic tension is virtually constant. Two amplitudes of shortening were used. The smallest release (the control) was sufficiently large to produce a complete drop in tension immediately followed by redevelopment of force. With the larger release (the test) the fibre was allowed to shorten over an additional distance before it started to redevelop tension. The releases were timed so that the redevelopment of tension occurred at the same

moment during the activity period in both test and control. The speed of release was high enough to prevent force production during the shortening phase.

Fig. 1 illustrates release recordings performed during a single twitch (A) and a fused tetanus (B) in the same fibre. It can be seen that the extra amount of shortening during the test release (0.16 $\mu\text{m}/\text{sarcomere}$) reduced the rate of rise and the peak amplitude of the redeveloped force. The time to peak tetanus was not markedly affected (see also Fig. 2, Edman 1975) but there was a certain (approximately 15%) reduction of the time to half relaxation. The depression of peak force in percent of the control, was 29.7% during the single twitch. A considerably smaller (4.0%) depression was obtained when the same amount of shortening was produced during the plateau (prior to the last stimulus) of the fused tetanus. Table 1 summarizes the results of six experiments similar to that illustrated in Fig. 1. In these experiments a sarcomere shortening of 0.14–0.46 μm was required to reduce the peak redeveloped force by 4.0–7.5% during a fused tetanus. The same amount of movement performed during the rising phase of a single twitch or during the last cycle of a partially fused tetanus reduced the peak redeveloped force by 25–51% in the same fibres.

The depressant effect of shortening during a fused tetanus is further illustrated in Fig. 2. In this experiment the stimulation was continued for about 800 ms after the end of the shortening. It can be seen (inset, Fig. 2) that the speed of force redevelopment (dF/dt) was lower at any given tension level during the first few hundreds of milliseconds after the large movement. The value of dF/dt at 50%

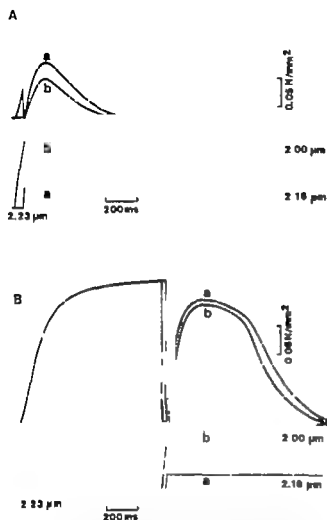


Fig. 1. Depressant effect of actin shortening during a single twitch (A) and a fused tetanus (B) in an isolated muscle fibre. The time of release was adjusted appropriately to allow the fibre to redevelop tension at nearly the same time after the small ($0.07 \mu\text{m}$ /sarcomere, myogram a) and the large ($0.3 \mu\text{m}$ /sarcomere, myogram b) movement. Lower traces: position of pulley arm indicating sarcomere length before and after the shortening. Note (1) that the force redevelopment is smaller after the large movement and (2) that the reduction of force is more pronounced during the twitch than during the fused tetanus. Temperature: 0°C . Cross-sectional area: $11.589 \times 10^{-6} \text{ mm}^2$.

mounting of the fibres have been described previously (Edman 1975, 1979). The fibres were mounted horizontally in a jacketed Perspex bath between a force transducer and the tip of a shaft which was connected to the moving coil of an electromagnetic puller. The rest length of the preparation could be set to the desired value by adjusting the position of the puller or the tension transducer.

Solution. The bathing solution had the following composition (mM): NaCl 115.5, KCl 2.0, CaCl_2 1.8, Na phosphate buffer ~ 0 , pH 7.0. The solution (8 ml) was ex-

changed before each series of contraction. The temperature was controlled by circulating a water-glycol from a thermostatically controlled tank (Cotara Thermostat) through the jacket surrounding the chamber. bath temperature was maintained constant to $\pm 0.1^\circ\text{C}$ during the experiment; the temperature varied between $\pm 0.5^\circ\text{C}$ between different experiments.

Stimulation. The fibres were stimulated by current through two platinum plate electrodes placed either side of the fibre approximately 1 mm apart. Rectangular pulses of 0.1 ms duration were used as stimulus. Stimulus strength was approximately 1.5° above threshold. A single pulse, a series of 4–6 pulses of 3–5 Hz frequency or a train of pulses of a frequency of 1 Hz were given to produce a single twitch, an incompletely fused tetanus or a fused tetanus, respectively. Contractions were produced at 1 min interval during the experiment. The fibres were usually mounted for 4 h before the experiment began and were tetanized periodically during the time. The contractile performance was usually stable a whole day of experimentation. Deterioration of the fibre was detected by decreased rate of rise of tension in the fused tetanus, tension drop during the tetanus and/or increased dispersion of sarcomere length as reflected by a widening of the first-order line of the diffraction pattern.

Tension recording. An RCA 5734 mechanoelectric transducer was used with a tubular glass extension hook for connecting the fibre. The compliance of the extension and hook was $1.5 \mu\text{m}/\text{mN}$. The natural frequency of the tension transducer was approximately 1 kHz when the glass shaft was immersed in solution. Later experiments used a semiconductor strain-gauge transducer (AE 801 Akyses kapet Mikroelektronik) used. A hook of stainless steel wire was attached by glue to the tip of the transducer bar. The resonant frequency of the transducer submerged in the solution was about 1 kHz . There was no detectable creep of the transducer after a sudden unloading of the transducer.

Electromagnetic puller, servo system and displacement transducer. These were essentially the same as described previously (Edman 1975). The release movements were performed from the same starting point of the electromagnetic coil. In order to attain a given final length of the fibre after different steps the rest length of the fibre was adjusted appropriately. This could be done with an accuracy of $3 \mu\text{m}$ (0.1% of the fibre length, Edman 1975). For illustration of such an experiment (Fig. 1) the recording of the displacement transducer has been shifted photographically to show the actual sarcomere length before and after the release step.

Sarcomere length and α . The sarcomere length at rest and during activity was determined from the diffraction pattern. The change in sarcomere length occurred in response to a release during activity calculated from the change in overall fibre length and sarcomere length at the onset of the release movement.

Determination of fibre length and sarcomere length. The length between the insertions of the fibre to the supports was measured to the nearest 0.05 mm with a Stereo II microscope (6 or $10\times$ magnification) at the end of each experiment. Before this measurement the sarcomere

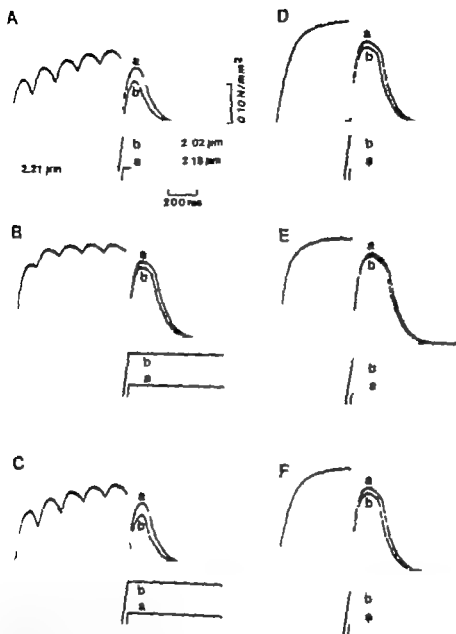


Fig. 1. Influence of caffeine on the depressant effect of active shortening. Control (a) and test (b) movements performed during the last cycle of partially fixed tetanus (A-C) and prior to the first stimulus of fixed tetanus (D-F). A, D: fibre immersed in ordinary Ringer solution. B, E, 45-65 min after addition of 0.5 mM caffeine to bathing fluid. C, F, 50-65 min after change to ordinary Ringer solution. Lower traces indicate sarcomere length before and after the shortening. In first stimulus marker in A and the first two markers in D have been retouched. Temperature: 25°C. Cross-sectional area: $4.877 \cdot 10^{-4} \text{ mm}^2$.

program the contraction was initiated at $2.20 \mu\text{m}$ and the fibre was allowed to shorten to $1.83 \mu\text{m}$ after which it was stretched back to its original length. The fibre's ability to produce force after a

complete drop in tension was tested at various times during cycles 2-6 in runs where no shortening (or stretch) had been performed during the first two cycles. For this purpose a small release (0.09

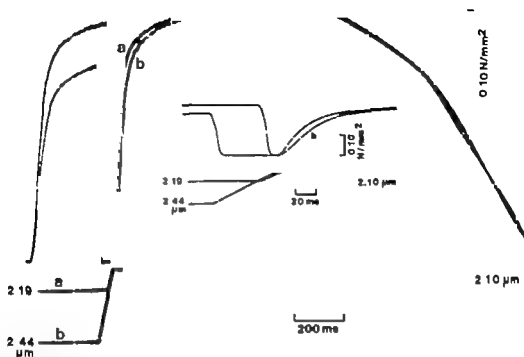


Fig. 2 Superimposed oscilloscope records illustrating redevelopment of tension after small (a) and large (b) shortenings during a fused tetanus. Inset: recordings on fast time base. Lower records, position of puller arm indicating sarcomere length before and after the shortening. Temperature: 12°C . Cross sectional area: $13.508 \times 10^{-6} \text{ mm}^2$.

of maximum tetanic force was 17% lower after the large movement than after the small control movement. The fibre regained its ability to produce force, however, so that almost the same tension level was attained after the large and the small movement at the end of the tetanus.

2 The influence of caffeine on the movement effect

The finding that shortening caused a smaller depression during a fused tetanus than during a single twitch or a partially fused tetanus suggested that the movement effect might be related to the degree of activation of the contractile system when the shortening occurs. As an attempt to further elucidate this possibility the depressant effect was studied after addition of 0.5 mM caffeine to the Ringer solution. This agent is likely to increase the amount of activator calcium in the myofibrillar space in response to excitation of the fibre membrane (e.g. Ebashi 1976; Blinks, Rudel & Taylor 1978).

Fig. 3 A-E shows that shortening caused a considerably smaller depression of the isometric force after 0.5 mM caffeine had been added to the bath. The depressant effect was 25% during the last cycle of the partially fused tetanus and 7% during

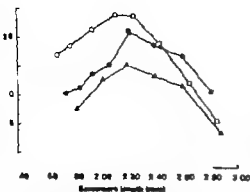
the completely fused tetanus in the ordinary Ringer solution. These effects were reduced to 7 and 1% respectively when the shortening was carried out in the presence of caffeine. Removal of the caffeine restored the original magnitude of the movement effect (Fig. 3 C-F). Results similar to those illustrated in Fig. 3 A-F were obtained in three other experiments.

3 Lack of effect of passive shortening

Shortening of the fibre before activation had no detectable effect on the subsequent course of contraction. This was tested by allowing the fibre to shorten passively from 3.05 to 2.60 μm sarcomere length immediately before the stimulus was applied. As can be seen in Fig. 4, twitches recorded at 4 μm with and without preceding passive shortening were identical in shape.

4 Tension development after shortening-restretch

Attempts were made to establish if restretch of the fibre after shortening reverses the depressant effect produced during the shortening phase. Fig. 5 illustrates such an experiment. Here are shown three myograms of partially fused tetani at 2.0 μm sarcomere length. In myogram b the fibre was allowed to shorten from 3.55 μm during the first cycle. I



6 Depressant effect of active shortening recorded various sarcomere lengths in three different fibres. Initial sarcomere length at which redevelopment of tension was measured after shortening. Ordinate: Depressant effect expressed in per cent of the control peak force each length. Sarcomere shortening during test run, in case of control release. \circ 0.24 $\mu\text{m/sarcomere}$, \square 0.25 $\mu\text{m/sarcomere}$, Δ 0.23 $\mu\text{m/sarcomere}$. Temperature and cross-sectional area, \circ 17°C $2.967 \cdot 10^{-4} \text{ cm}^2$, \square 16°C $6.980 \cdot 10^{-4} \text{ cm}^2$, Δ 15°C $4.604 \cdot 10^{-4} \text{ cm}^2$.

$\mu\text{m/sarcomere}$) was carried out at selected times and the peak redeveloped force was recorded at 2.0 μm sarcomere length. These data points (filled circles Fig. 5A) serve as controls for estimation of the amount of force depression during cycles 3–6 in myograms b and c.

The results in Fig. 5A show, in confirmation of previous results (Edman 1975) that shortening during the first cycle caused a marked depression of the contractile strength and that this effect gradually disappeared over a time period of approximately 800 ms, i.e. by the attainment of peak tension during the sixth cycle. Restretch of the fibre after an initial shortening phase did not reverse the depressant effect. In fact myogram c exhibits a somewhat larger force depression than myogram b. This difference may be due to the fact that the shortening in myogram c occurred over a range of sarcomere lengths where the depressant effect was greater than over the interval of lengths where the shortening was performed in myogram b (cf. length dependence of movement effect section 5).

Fig. 5B shows that prestretch of the fibre did not affect the depressant effect of shortening. The two myograms b and c were recorded at $\sim 0.2 \mu\text{m}$ sarcomere length in both cases after allowing the fibre to shorten from 2.22 μm during the first cycle. In myogram c the shortening was preceded by a

stretch from ~ 0 to 2 μm sarcomere length. As can be seen the time course of force development during cycles 3–6 was quite similar in the two runs.

5 The length dependence of the movement effect

The depressant effect of active shortening was studied at various sarcomere lengths in the range 1.7–2.9 μm . The experimental protocol was similar to that described in section 1. In the present experiments however the test and control contractions were initiated at different sarcomere lengths to allow the fibre to redevelop force at the same length after the small (control) and the large (test) release. The shortening was performed during the last cycle of a partially fused tetanus. The amount of shortening was 0.29–0.31 $\mu\text{m/sarcomere}$ during the test and 0.03–0.08 $\mu\text{m/sarcomere}$ during the control.

The results from 3 expts. are summarized in Fig. 6. The depressant effect has been expressed in per cent of the redeveloped force in the control run at each length. It can be seen that for a given amplitude of shortening the force depression was maximum when the shortening ended at 2.1–2.2 μm sarcomere length. The depressant effect decreased when the shortening was performed at longer and shorter lengths. The mechanical behaviour of a given fibre was consistent during repeated contractions at various sarcomere lengths. It is evident from Fig. 6 however that the length dependence of the movement effect exhibits a somewhat greater variability from fibre to fibre than observed for the relationship between tetanic force and sarcomere length (see Fig. 1 Edman 1966 and Fig. 3 Gordon, Huxley & Julian 1966).

6 The effect of shortening on the force-velocity relationship

The following experiments were performed to find out if the depression of isometric force that occurs in response to active shortening is paralleled by a decrease of the maximum velocity of shortening. The same general approach was used to study the depressant effect as described in Fig. 1A, i.e. the redevelopment of force was recorded after the fibre had been allowed to shorten by a small and a large amount during the activity period. The velocity of unloaded shortening (v_u) was determined during the rising phase of the redeveloped twitch using the multiple-release method described previously (Edman 1979). The results of 1 expt. are illustrated

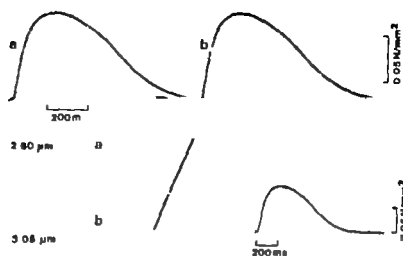


Fig. 4. Two isometric twitches recorded with a time difference of one minute. Record *a* no preceding shortening. Record *b* contraction preceded by passive shortening. The higher base line (resting tension) under myogram *a* refers to the situation prior to release in myogram *b*. Inset: record *a* and *b* repeated and superimposed upon each other on oscilloscope screen. Temperature 19°C . Cross-sectional area $5.604 \times 10^{-4} \text{ mm}^2$.

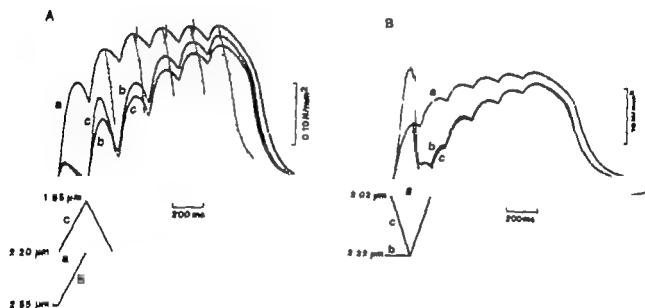


Fig. 5. (A) Isometric force development at $2.20 \mu\text{m}$ sarcomere length during incompletely fused tetanus in the absence (myogram *a*) and in the presence of shortening (myogram *b*) or shortening restretch (myogram *c*) during the first two cycles. Filled circles (connected by thin line) indicate peak redevelopment of tension at $2.20 \mu\text{m}$ sarcomere length at a small ($0.09 \mu\text{m}$ /sarcomere) release carried out at different times during cycles 2-6. *m* in the absence of shortening restretch during cycles 1-6. Arrows indicate tension values used as control for the peak twitch force during cycles 3-5 of myograms *b* and *c*. Lower records (retracings) position of puller arm indicating sarcomere length during myograms *a-c*. Temperature 16°C . Cross-sectional area, $13.914 \times 10^{-4} \text{ mm}^2$. (B) Force development at $2.02 \mu\text{m}$ sarcomere length of incompletely fused tetanus in the absence (myogram *a*) and in the presence of shortening (myogram *b*) and stretch-shortening (myogram *c*) during the first two cycles. The three myograms have been superimposed photographically. Temperature, 13°C . Cross sectional area, $9.791 \times 10^{-4} \text{ mm}^2$.

Table 2. The decline of unloaded shortening (% Δ) measured during single twitch after depression of the active force by active movement

| | Temp °C | % of control twitch (L/sec) | % of depressed twitch (% control) | Peak force of depressed twitch (% control) |
|--------------------|------------|-----------------------------------|---|--|
| 21 1978 | 11 | 2.32 | 100 | 84 |
| 1979 | 0.7 | 2.19 | 96 | 58 |
| 1979 | 0.4 | 2.19 | 97 | 68 |
| 1979 | 1.4 | 2.16 | 103 | 50 |
| \pm S.E. of mean | | | 99 \pm | 65 \pm 7 |

movement effect. The Q_{10} based on 3 expts.
is 95 ± 0.16 (S.D.)

DISCUSSION

The kinetics of the movement effect

The present study has provided further evidence that active shortening involves some change in the contractile system that reduces the fibre's ability to produce force over a relatively long time (800–900 ms) after the movement. The results have shown that this depressant effect appears during a fused tetanus as well as during a twitch or a partially fused tetanus. It can furthermore be concluded that the phenomenon is related to a shortening movement only (cf. Edman, Elzinga & Noble 1978): a stretch performed immediately before or after the shortening (at sarcomere lengths $< 2.25 \mu\text{m}$), was found to have no significant influence on the depressant effect. Deactivation by shortening has also been shown to occur in amphibian slow muscle fibres (Lillemor 1978), mammalian skeletal muscle (Joyce & Rack 1969; Joyce et al. 1969) and amphibian and mammalian myocardium (Brady 1966; Edman & Nilsson 1971; Bozler 1972; Kaufmann, Bayer & Harnasch 1972; Bodem & Sonnenblick 1975). I.e. the phenomenon would seem to apply for striated muscle in general. This finding is fully in line with the view (see below) that the movement effect is a specific consequence of the sliding-filament process under conditions normally existing in the living fibre.

There is evidence that the magnitude of force depression after shortening is dependent on the state of activation of the fibre when the shortening occurs. The decrease in isometric force for a given amplitude of shortening was thus found to be small-

er during a tetanus than during a twitch. The effect was also reduced after treating the fibre with calyculin which can be presumed to increase the concentration of calcium in the myofibrillar space (Ebashi 1976; Blümlin et al. 1978). These observations accord with the previous finding (Fig. 4; Edman 1975) that the depressant effect is steadily increased as the shortening is carried out at later times during a twitch when the activity decays.

The movement effect described here should be distinguished from the decrease in contractile strength that occurs when an excessive amount of work is produced during contraction. An increase of the work performance above approximately $2 \mu\text{J}/\text{cm}^2$ (Edman & Mattiazzi unpublished results) causes a decrease of the tetanic force which persists during continued (4–6 s) stimulation of the fibre (Abbott & Aubert 1952; Edman 1966; Maréchal & Plakol 1979). This force deficit increases with the amount of work produced. By contrast, the movement effect described in the present paper is not significantly dependent on the force or the work output during the shortening phase (Fig. 2; Edman 1975). Furthermore, full contractile strength is regained during continued isometric activity. These features of the movement effect suggest that the transitory decrease in mechanical performance that is produced by shortening is not due to exhaustion of chemical energy for the contractile process. Evidence obtained in studies of ATP and creatine phosphate breakdown during unloaded shortening supports this view (Abbott & Howarth 1973; Curtin & Woledge 1978; Homsher & Kean 1978).

Curtin & Woledge (1978) direct attention to the possibility that the movement effect might bear some relation to the shortening heat, i.e. the extra

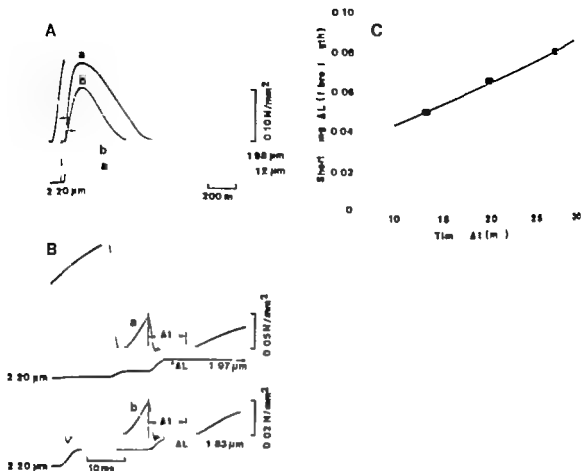


Fig. 7. Measurement of force and unloaded velocity of shortening (v_u) after active movement during single (A) and release recordings (B). (A) Redevelopment of tension after small (0.08 $\mu\text{m}/\text{sarcomere}$; myogram a) and large (0.2 $\mu\text{m}/\text{sarcomere}$; myogram b) movement. (B) Release recordings for determination of v_u carried out after small (myogram a) and large (myogram b) movement at times indicated by arrows in A. ΔL , amplitude of release in v_u analysis. Δt , time from onset of release to beginning of force redevelopment. Note different tension scales for myograms a and b. (C) Relationship between ΔL and Δt in control twitch (●) and in twitch depressed by large movement (○). Each point is the mean of three separate recordings. The slope of the line relating ΔL to Δt (regression analysis) is 2.19 and 1.12 $\mu\text{m}/\text{sec}$ in control and test twitches, respectively (regression coefficients 0.996 and 0.989; $n=9$). Temperature: 0.4°C . Cross-sectional area: $3.659 \times 10^{-4} \text{ cm}^2$.

In Fig. 7. In this experiment the large movement depressed the peak isometric force to 58% of the control value (Fig. 7A). Examples of release recordings used for determination of v_u after the small and the large movement are shown in Fig. 7B. As can be seen, the time (Δt) from the onset of release to the onset of force redevelopment was very nearly the same in the two cases. The complete analysis of v_u , based on three different amplitudes of release (ΔL), is shown in Fig. 7C. The slope of the line relating Δt to ΔL was not significantly different in the depressed twitch and the control twitch. The results of four experiments including that illustrated in Fig. 7 are summarized in Table 2. It can be seen that whereas the force was depressed to 50–84% by active shortening in the different

experiments, v_u remained unaffected to within 4% of control.

7. The influence of temperature on the movement effect

In order to determine the temperature dependence of the movement effect, the speed of force redevelopment (df/dt) after shortening was studied during tetani at 1.50–2.00 and 11.25–11.38 $^\circ\text{C}$. By the approach illustrated in Fig. 2 (inset), it was possible to compare the movement effect at two different temperatures at maximum degree of activation of the fibre. For determination of the depressant effect, df/dt was measured in both test and control at 50% of maximum tetanic force. A rise in temperature did not cause any significant change

size of the movement effect, with a maximum at 2.1 μm sarcomere length does not reflect the degree of activation of the contractile system at various sarcomere lengths. Results reported by Huxley & Edman (1978) suggest that the amount of calcium released by the action potential is maximum between 2.4 and 3.0 μm sarcomere length in different fibres and that the calcium release is steadily reduced at shorter and more extended lengths. The length dependence of the depressant effect would thus seem to be governed by a factor that over-rides the influence of activation.

The interesting possibility emerges from these results that the relative position of the A and I filaments during the movement somehow determines the magnitude of the depressant effect. It is evident from Fig. 6 that the depression of force does not change in proportion to the control isometric tension as the overlap area between the thick and thin filaments is altered. The results could mean that the myosin bridges are progressively effective in deactivating the contractile system the closer to the centre of the thick filament the bridges are located; in this case the movement effect would rise to a maximum as the sarcomere spacing is reduced to 1 μm . The effect would decline below this point if filaments intruding from the opposite half of the sarcomere would prevent the centrally located bridges from interacting with the same side A filaments.

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heat produced during active shortening and that is not explained in terms of ATP splitting or the creatine kinase reaction. The heat of shortening and the movement effect are both largely independent of the speed of shortening; they increase with the amplitude of shortening and they both reach a maximum at the plateau of the length-tension diagram (cf. Curtin & Woledge 1978). Other observations, however, make it seem unlikely that the two phenomena are causally related. Thus the heat of shortening has been found to decline progressively when contractions with shortening are repeated at 5 sec intervals (Dickinson & Woledge 1974). No such dependence on the previous activity of the muscle applies for the force depression after shortening.

The movement effect does not include a change of v ; the measured velocity of shortening at zero load. This accords with the observation that the speed of shortening against a small load ($P < 0.01 P_0$) remains very nearly constant as the fibre shortens between 2.4 and 1.7 μm sarcomere length under load-clamp conditions (A. A. P. Edman unpublished data). The constancy of v also agrees with the previous finding (Edman 1979) that v is independent of the fibre's capacity to produce force within the range $P_0/2$ to P_0 . The lack of effect on v would seem to rule out that shortening produces a passive resistive force that accounts for the decrease in contractile strength after the movement. An increase of the intrinsic load would cause a relatively large decline of v ; considering the steep relationship between force and velocity of shortening at loads close to zero.

Nature of movement effect

Deactivation. The relatively long duration of the movement effect (nearly one second) suggests that this phenomenon is distinct from the mechanical transients that occur after a quick length change (stretch or release) of an active muscle fibre (Podolsky 1960; Huxley & Simmons 1971). The mechanical transients, which involve changes of both tension and shortening velocity, can be explained in terms of the kinetic properties of the cross-bridges (Civan & Podolsky 1966; Huxley & Simmons 1971; Bressler & Clinch 1974; Ford, Huxley & Simmons 1977) and are not likely to reflect an altered state of activation of the contractile system. The movement effect, on the other hand, has features that are characteristic of deactivation (Edman

1979), i.e. the force output is reduced without change of v .

In explaining the deactivating effect of shortening, the following mechanisms may be considered:

1. Increased rate of sequestration of activator calcium by the sarcoplasmic reticulum.

2. A structural change in the contractile system that leads to a reduced actin-myosin interaction.

The first alternative appears unlikely in the light of the present results. Such a mechanism would require that shortening increases the effectiveness of the calcium pump of the sarcoplasmic reticulum and that this effect persists over several hundred milliseconds after the movement. In such a case a passive shortening performed immediately before stimulation would be expected to affect the subsequent course of contraction. The results have shown, however, that the shortening has to occur during activity in order to reduce the contractile strength.

In considering the second alternative, the temperature dependence of the movement effect, with a Q_{10} close to 1, is consistent with a change of a protein structure. The precise nature and location of the structural change in the myofilament system cannot yet be defined, however. It has previously been suggested (Edman 1975) that active shortening causes some change of the regulatory protein complex of the I filament that leads to a transitory impairment of the actin-myosin interaction. The structural change may decrease the affinity of the binding sites for calcium and thus reduce the amount of activator calcium in the contractile system (cf. Edman & Hoesling 1971 and Kaufmann et al. 1977). Another possibility worth taking into account is that shortening produces a structural change or dislocation of a fraction of the myosin bridges, thereby decreasing the ability of these bridges to interact with the actin filament.

The idea that shortening reduces the concentration of calcium in the myofilament system is attractive as it conforms with the finding that the movement effect is less pronounced at a higher degree of activation of the fibre, i.e. when the concentration of calcium is increased. The fact that shortening causes some abbreviation of the mechanical activity (Fig. 1) is also in line with the hypothesis that calcium is mobilized from the contractile system during the shortening phase.

The length dependence of movement effect. There is reason to believe that the length dependence

ence of the movement effect, with a maximum near $1\text{ }\mu\text{m}$ sarcomere length does not reflect the degree of activation of the contractile system at various sarcomere lengths. Results reported by Blinns et al (1978) suggest that the amount of calcium released by the action potential is maximum between 2.4 and $3.0\text{ }\mu\text{m}$ sarcomere length in different fibres and that the calcium release is steadily reduced at shorter and more extended lengths. The length dependence of the depressant effect would thus seem to be governed by a factor that overpowers the influence of activation.

The interesting possibility emerges from these results that the relative position of the A and I filaments during the movement somehow determines the magnitude of the depressant effect. It is evident from Fig. 6 that the depression of force does not change in proportion to the control isometric tension as the overlap area between the thick and thin filaments is altered. The results could mean that the myosin bridges are progressively effective in deactivating the contractile system the closer to the centre of the thick filament the bridges are located. In this case the movement effect would rise to a maximum as the sarcomere spacing is reduced to $2.1\text{ }\mu\text{m}$. The effect would decline below this point as I filaments intruding from the opposite half of the sarcomere would prevent the centrally located bridges from interacting with the same side I filaments.

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Topical application of penicillin into hippocampal in vitro slices. A methodological study using benzyl (¹⁴C) penicillin

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OLOFSSON P Å RIDDERHEIM P Å & SILFVENIUS H Topical application of penicillin into hippocampal in vitro slices. A methodological study using benzyl (¹⁴C) penicillin. *Acta Physiol Scand* 1980; 109: 77-86. Received 26 July 1979. ISSN 0001-6772. Departments of Physiology and Neurosurgery University of Umeå, Sweden.

A gas pressure system is employed for topical application of pico/nanolitre volumes of 9 mmol/l benzyl (¹⁴C) penicillin into guinea-pig in vitro transverse hippocampal slices. Control of pressure pulse parameters enables ejection, compressed after liquid desiccation, from 18 µm 5 µm and 2 µm pipettes with high reliability of ejection. A detailed study was done on the ejection performance of 5 µm and 2 µm pipettes. It shows that for the 5 µm tips with the chosen ejection parameters, feeding pressure = 0.5 MPa, feeding pulse duration 70 ms, a 99% ejection incidence was obtained (> 200). The mean volumes ejected were 0.12 and 0.5 nl (± 30) for one and four pulses delivered respectively. The corresponding ejection masses obtained from five 2 µm pipettes given a feeding pressure of 1.5 MPa, and a feeding pulse duration of 70 ms, were 0.04 and 0.13 nl. Statistical evaluation of the individual pipette ejection performances gave characteristic regression slopes within each pipette group. A comparison between visually controlled injection into old and injection into slices showed close correspondence. The applicability of the ejection method is discussed in relation to functional studies on the conversion of single neurones into epileptic ones.

Key words: Benzylpenicillin, radioactive pressure microinjection, in vitro hippocampal slice

Benzylpenicillin has since long been used as an epileptogenic agent in experimental studies. Topical application of the substance onto single cells may be made from micro pipettes using either mechanical, iontophoretic or thermal methods. Studies on the effect of benzylpenicillin on single neurones, a quantification of the topically applied substance is desirable since functional alterations evoked should preferably be correlated to the ejection volume as well as to the exact site of application. A reasonably accurate method of quantification of small amounts of ejected benzylpenicillin is therefore required. Such a method could lead to the determination of the neuronal membrane region contacted by the ejected substance. Applied extracellularly to neocortex benzylpenicillin alters the neuronal discharge pattern in the incomplete negative circuit towards excitation (Walsh 1971; Curtis et al. 1972). The present report describes method of pressure extrusion of benzylpenicillin from glass

micro pipettes. It displays as will be shown a high degree of reliability showing only moderate scatter between the individual ejection as evidenced by the amounts of ¹⁴C-benzylpenicillin, injected into guinea-pig transverse hippocampal in vitro slices. The method will therefore later be applied to study the effect of benzylpenicillin on single hippocampal cells.

METHODS

Transverse hippocampal slices, 400 µm thick, were cut from guinea-pigs weighing 300-500 g, anesthetized with chloroform, in a box into which a 95% O₂-5% CO₂ gas mixture was led at 4 l/min. The slices were maintained in vitro according to the method of Skrede & Westgaard (1971). A stock solution of µCi/µl benzyl (¹⁴C) penicillin (S.A. 33 mCi/mmol 2.0 GBq/mmol, Amersham, England) was prepared with absolute ethanol. A certain amount of the solution was further diluted 1:3 while the rest was evaporated under N₂ gas and stored at -20°C. Eleven glass tubes were pipetted with 10 µl of the 1:3 dilution. One

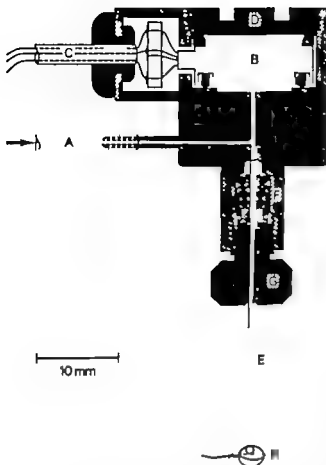


Fig. 1 Schematic side view of the Aluminium pipette holder used for pressure ejections of ^{14}C -penicillin. A Impolen tubing connected to the magnetic valve; arrow indicating feeding pressure wave inflow. B Miniature pressure transducer measuring pulse waves (see Fig. 3A and 4A) connected via C to amplifier system and screwed into holder by screw D. E ^{14}C -penicillin glass pipette. F Inset piece to minimize angling of pipette. G Screw cap for pipette with O-rings. H Oil droplet in silver loop and a penicillin spherule.

of them was further diluted to $1000\ \mu\text{l}$. Of this dilute $10\ \mu\text{l}$ was transferred to a scintillation vial which radioactivity was determined. This served as reference $\times 1000$ for the other 10 glass tubes pipetted. They were evaporated under N and stored until the day of expt.

$10\ \mu\text{l}$ of filtered hippocampal slice bath medium was then added to a tube used in one expt. solving the ^{14}C -penicillin. From the $10\ \mu\text{l}$ solution a small amount was sucked into a thin glass capillary drawn over heat by hand from a 2 mm diameter glass tube. This capillary was inserted into the shaft region of a GC 170F glass pipette (Clark Electromedical Instrs. England) drawn to a tip that was broken to a particular size $\sim 10\ \mu\text{m}$. A ^{14}C -penicillin pillar about $300\ \mu\text{m}$ above shaft level was usually kept in the pipettes. All air bubbles were allowed to escape from the pipette solution.

The ^{14}C -penicillin concentration was $9\ \text{mmol/l}$. The ^{14}C -penicillin pipette was then tightly screwed into it. A holder of the pressure injection system. This consisted

of (cf. Fig. 1): a) a pressure source with regulator connected with an Aluminium embedded tubing (Delat 1300" Samuel & Moore USA) to b) a magnetic way valve (Lucifer Switzerland) of 9.4 or 2.5 MPa nominal capacity and with a response time of 15 ms of 90 feeding pressure. The valve was driven by c) a pulse generator with stepless duration manoeuvring and connected to the d) pipette holder with a polypropylene tubing (Impolen 22-PP ITE USA). The holder contained e) a miniature pressure transducer (either type AI 830 D or type AF 831 F (AS Mikro Elektronik, Norwa) with ranges of 0–1 and 0–5 MPa being in air contact at the top of the ^{14}C -penicillin fluid pillar. The output for the transducer was fed via an f) amplifier system to a beam on the CRO. Pressure pulses recorded by the transducer are shown in Figs 3A and 4A. The peak pressure P_p and feeding pressure P will in the following be referred to.

The ^{14}C -penicillin pipette and its holder was carried by micromanipulator driven by a stepmotor. Under visual microscopic control the pipette was advanced in the slice chamber until its tip mirrored on a slice surface. It was then further advanced at an angle of 10 degrees to the vertical plane to a depth of $150\ \mu\text{m}$ in the stratum radiatum of the CA hippocampal region. The ejection was done at this depth.

Except in early expts. identical pressure pulses were given $1\times$, $2\times$, $3\times$ and $4\times$ and a series of ten slices were injected at each pressure group. The reliability and ejection scatter of the method could thereby be assessed.

Between slice ejections the pipette was cleaned in the moist gas of the chamber and the ^{14}C -penicillin droplet extruded was sucked off with the wet tip of a tissue paper roll. The possible remaining contamination of the wiped tip never resulted in above background detectable counts even if the tip was kept in a slice by giving no pressure pulse for periods more than 5 min.

In the course of the experiments the ejection method was modified. Prior to injecting into a slice an ejector was made into a tiny oil droplet kept in a $300\ \mu\text{m}$ diameter thin silver wire loop. The ^{14}C -penicillin spherule ejected into oil could clearly be visualized in the viewing microscope if of a diameter $>10\text{--}15\ \mu\text{m}$. Usually the P was adjusted so that the ^{14}C -penicillin spherule in oil had diameters of $80\text{--}100\ \mu\text{m}$ corresponding to $0.014\text{--}0.02\ \mu\text{Ci}$. The pressure adjustments were made for each pipette.

The tip sizes used were between ~ 5 and $10\ \mu\text{m}$. The tip size was checked after the expt. and found unchanged.

Blow outs were seen in slices leaving tiny temporary craters. If large pipette tips were used together with high pressure pulses $\geq 10\ \mu\text{m}$ tips, P around $0.5\ \text{MPa}$. After injection the pipette was withdrawn and the injected slice was removed from the bath with metal spatula into a scintillation vial containing 1 ml of tissue solubilizer (Sciencelab 100 Packard USA). The injected slices were allowed to disintegrate at $+40^\circ\text{C}$ for 30 min after which 1 ml of scintillation liquid was added to each vial.

The vials were counted after equilibration in a Liquid Scintillation Spectrometer (Packard 3310) for 5 min. Occasional series were counted 5 min, 10 or 20 to assess scintillation variability. Counts were converted to 100 times expressed a pl or nl.

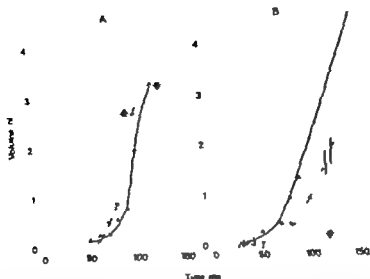


Fig. 2. A. Response curves of 10 μ m pipette from which 14 C-penicillin was ejected at feeding pressure, P , of 49 kPa during increasing (●-●) and decreasing (▲-▲) pulse lengths. Each dot at shortest refers to the ejectate in one slice. B. Pressure-response curve of a 10 μ m pipette (▲-▲) through which 99 kPa pulses of 30-150 ms durations were given, being compared with that of 5 μ m pipette (●-●) tested on similar pressure parameters. The latter one was blocked at pulse duration 120 ms. Each value refers to 14 C-penicillin ejectates from one slice. Free hand fitting of curves.

Quenching as checked by comparing counts obtained in vials containing Solonox 100 and scintillation liquid and such also which non-ejected slices or the first liver thread loops had been dropped. Differences above background values were not obtained.

Statistical evaluation and plotting was done using TATPAC for NORU-10 computer programmed at the Järnkärr Institute, Stockholm.

RESULTS

The quantification of pressure ejected 14 C-penicillin from glass micro pipettes into the CA stratum radiatum of guinea-pig hippocampal *in vitro* slices was made using a number of pipettes with tip sizes 5 and 10 μ m. Initially mainly single injections were made during which the feeding pressure, P , was kept constant while driving pulse, duration T , was altered. Low P injections were made with 5 and 10 μ m tips.

The lowest P tested on 10 μ m pipettes was 29 kPa. With T pulses of 50-100 ms durations, above background detectable radioactivity was found but almost every other slice contained no detectable radioactivity. When the T was increased to 120-150 ms ejectates between 0.16-1.1 nl were obtained. An increase in P to 49 kPa gave a more

reliable dose-response curve for 10 μ m pipette as is shown, in Fig. 1A.

For this particular 10 μ m pipette the P pulses were first given in an increasing T duration sequence (●-●-●) and then in the opposite direction (▲-▲-▲). As seen in Fig. 2A both sequences gave similar slopes of the curves with different ejectates at the individual pulse duration steps. In order to obtain more data on the relation between P and T at low P values nine 10 μ m pipettes were tested at the P levels 29, 49 and 59 kPa, using T between 30-150 ms. The individual 14 C-penicillin ejectates varied somewhat as is seen in Table 1A.

With a P of 29 kPa and with T pulses between 30-150 ms of the twenty-six injections given ten slices contained extremely small amounts (0.01-0.02 nl) suggesting that in 39% there was complete or partial blocking of the pipettes.

In any of the three pipettes tested at this P the small ejectates could occur at any T duration. Thus despite variations in volume there was a positive correlation between T and nl in each pressure group. Increasing the P to 49 kPa gave a more reliable outcome of 14 C-penicillin ejectates from the three 10 μ m pipettes tested. In this injection series there were no borderline ejectates only three

Table 1 Amounts of ^{14}C penicillin nl in hippocampal slices obtained with low P_i s at varying driving pulse durations

| Driving pulse duration | | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 150 ms |
|---|-----|------|------|------|------|------|------|------|--------|
| A 10 μm pipettes | | | | | | | | | |
| I 29 kPa | 1 | 0.01 | 0.37 | 0.01 | 0.38 | 0.8 | 0.0 | 0.01 | 0.01 |
| | | 0.16 | 0.14 | 0.0 | 0.01 | 0.11 | 1.07 | 1.61 | 2.99 |
| | 3 | 0.0 | 0.06 | 0.03 | 0.0 | 0.09 | 1.5 | 1.94 | 1.9 |
| Mean | | 0.07 | 0.19 | 0.02 | 0.14 | 0.16 | 0.78 | 1.18 | 1.31 |
| II 49 kPa | 4 | 0.17 | 0.76 | 0.59 | 0.79 | 1.97 | 5.0 | 3.28 | 4.10 |
| | 5 | 0.11 | 0.1 | 0.04 | 0.03 | 0.70 | 1.1 | 63 | 0.06 |
| | 6 | 0.15 | 0.19 | 0.33 | 0.57 | 1.15 | 33 | 08 | 5.31 |
| Mean | | 0.14 | 0.19 | 0.32 | 0.45 | 1.27 | 2.89 | 2.87 | 3.16 |
| III 59 kPa | 5 | 0.19 | 0.3 | 0.77 | 0.88 | 1.71 | 3.67 | 1.99 | 4.51 |
| | III | 0.46 | 0.54 | 0.64 | 1.10 | 1.66 | 3.38 | 3.91 | 5.46 |
| | 7 | 0.5 | 0.16 | 0.41 | 0.96 | 1.31 | 2.81 | 58 | 4.9 |
| Mean | | 0.30 | 0.34 | 0.59 | 0.98 | 1.56 | 3.79 | 3.49 | 4.83 |
| B 5 μm pipettes | | | | | | | | | |
| I 59 kPa | 1 | 0.15 | 0.05 | 0.08 | 0.4 | 1.1 | 0.9 | 0.00 | 3.3 |
| | 2 | 0.07 | 0.00 | 0.14 | 0.12 | 0.16 | 0.30 | 0.69 | 0.88 |
| II 78 kPa | 3 | 0.07 | 0.11 | 0.12 | 0.14 | 0.40 | 0.73 | 0.75 | 0.68 |
| | 4 | 0.04 | 0.11 | 0.00 | 0.1 | 0.34 | 0.56 | 0.90 | 0.93 |
| III 98 kPa | 5 | 0.14 | 0.19 | 0.45 | 0.66 | 1.16 | 1.71 | 1.60 | 1.20 |
| | 6 | 0.06 | 0.11 | 0.10 | 0.19 | 0.5 | 0.48 | 0.54 | 0.43 |

slices contained ejectates distinctly lower than the other (i.e. 0.03–0.06 nl). They were all obtained from one pipette. The largest ejectates obtained at P_i 49 kPa with pulse duration extremes of 50 and 150 ms were 0.17–5.31 nl. Elevating the P_i further to 59 kPa increased the repeatability of injections; the small ^{14}C penicillin amounts lacking in this series of three 10 μm pipettes. The largest ejectate volumes obtained at this P_i level with pulse extremes of 50 and 150 ms were 0.46 and 5.46 nl. Expectedly least interpipette scatter between individual ejectates was obtained at this P_i .

Thus low pressure injection of ^{14}C penicillin with the present system into hippocampal slices was obtained with acceptable but somewhat variable yields provided the P_i was of the order of 50–60 kPa and the driving pulse duration preferably exceeds 50 ms. Clogging of the tip occurred often if lower P_i s were used.

In order to obtain some piece of information on the ejection performance of 10 μm vs 5 μm pipettes a P_i of 59 kPa was fed into two pipettes of either size. The outcome of this comparison is shown in Fig. 2B. T_d s of the order of 30–60 ms gave practically no detectable amounts of ^{14}C -penicillin in the

two slices. Pulses 70 ms or longer yielded ejectates which except for one clogging incidence increased similarly but of different volumes. In the particular experiment the 5 μm pipette was further kept in an additional slice for 25 min with a pressure pulse being given. It was found that 0.06 nl of ^{14}C penicillin had diffused out from the tip during that period.

Ejectates obtained from six 5 μm pipettes are further listed in Table 1B. The lowest P_i tested, 59 kPa, was equal to the highest tested P_i on 10 μm pipettes. Comparing the 5 μm and 10 μm pipettes it was found that the general yield at this P_i is comparable to P_i s for the 10 μm pipettes of about 50 kPa. Of the nineteen injections given at P_i 59 kPa measurable amounts for the two 5 μm electrodes were obtained in sixteen i.e. about 84% of presumed clogging occurred (cf. Table 1B I). Increasing the P_i to 78 kPa the reliability of ejectates increased together with a decrease in interpipette scatter (cf. Table 1B II). A III given to a 5 μm pipette identical in duration but twice of that delivered to a 10 μm pipette gave thus on the whole comparable ^{14}C -penicillin ejectates (compare Table 1A II with 1B III).

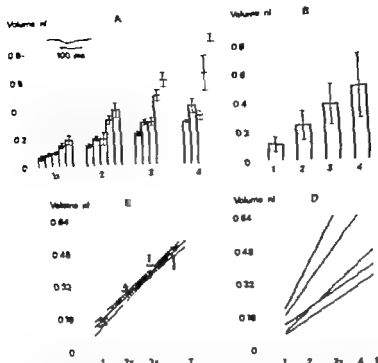


Fig. 3. A Histogram showing means and S.D.s of five 5 μ m pipettes from which 14 C-penicillin was ejected. Each column corresponds to ten slices. The individual pipettes were pressure ejected 1–4 with identical pulses, the feeding pulse being 70 ms and 0.50 MPa. The actual pulse pressure length was 150 ms, the rise time 20 ms. The peak pressure P_m was 0.20 MPa. Undetectable amounts were found in 2 out of 200 slices. Inset shows pressure pulse recorded by transducer as described in Fig. 1B. From 3A, pooled 14 C-penicillin ejection. Means and S.D.s are shown. Dots at abscissa in A and B refer to 'zeros' found at one and two pulse injections. The zeros are included in calculations. C Linear regression plot for individual volumes of 14 C-penicillin ejection obtained from one 5 μ m pipette, graphically represented by the third column in each injection group in Fig. 3A. A 95% confidence interval for mean of volumes is drawn. D Linear regression slopes of fifty volumes obtained at each ejected pressure number group from five 5 μ m pipettes.

14 C-penicillin ejection from 5 μ m pipettes with pressure ejections (60–100 kPa) yielded an outcome in 95% but with a high scatter between the individual amounts. A doubling of the P (120–200 μ s) for 5 μ m pipettes should therefore be performed if ejection is to be obtained comparable to those from 10 μ m pipettes. In order to ascertain the accuracy to the test of the reliability of any 5 μ m pipette ejection performance the experimental procedure was modified in the following way. Prior to injecting into a slice a chosen P and T were set to obtain a 14 C-penicillin spherule in oil as described in METHODS. As the viewing microscope allowed accuracy of the 70 μ m diameter magnitude of the penicillin spherule in oil, the P and T were set

to produce a 14 C-penicillin spherule of about 80–100 μ m diameter corresponding to about 0.5 nl. Once the pressure pulse parameters were set for each individual 5 μ m pipette they were left unaltered. The only parameter being changed was the number of pulses given. Depending on the ejection characteristics of the individual 5 μ m pipettes the size of the obtained 14 C-penicillin spherule in oil varied between the pipettes. The reliability of the individual pipettes was then tested by giving identical pulses 1x, 2x, 3x and 4x to ten different slices in each pulse repetition group. Forty slice ejection were thus obtained from each pipette.

The outcome of the test performance on five different 5 μ m pipettes with a P of 0.50 MPa and P of

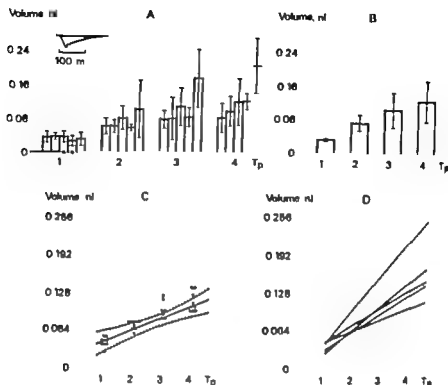


Fig. 4. A: Quantities of ^{14}C -penicillin encountered in 700 slices injected with a P of 1.5 MPa from five 2 μm pipettes with 1-4 pressure pulses of 70 ms. Pressure pulse wave measured at top of pipette shown in inset. The size of the test-drop of ^{14}C -penicillin in oil was 30-40 μm in diameter corresponding to about 30 pl. The peak pressure was 0.45 MPa. Means and SDs are shown. Each column refers to ten slices. Symbols at abscissa in two first group columns refer to undetectable amounts in four slices. B: Histograms from 4 A with means and SDs. C: Linear regression plot from one 2 μm pipette represented by the fourth column in 4 A, showing distribution of volumes and 95% confidence interval for mean of volume. D: Linear regression slopes of fifty volumes obtained at each pressure number groups ejected from five μm pipettes.

0.70 MPa and a driving pulse of 70 ms on totally 700 hippocampal slices (fifty slices in each pressure group) is shown in Fig. 3A.

The histograms of Fig. 3A indicate that the used pressure parameters on 5 μm pipettes secure a high repeatability in ejection performance: clogging of the tip occurring only in 1%. If one or two pressure pulses are delivered (Fig. 3A) further illustrates that the individual pipettes have their own characteristic ejection slopes related to delivered pulses. If the five injection series are pooled together, the means and SDs obtained from injections into fifty slices at each number of pulses are shown in Fig. 3B. From these data it can be concluded that one pressure pulse secures in 99% of the slice injections an average volume of 0.17 nl ^{14}C -penicillin.

Data on the distribution of individual ^{14}C -penicillin ejection volumes from a single 5 μm pipette are shown with a linear regression plot and a 95% confidence interval for mean volume in Fig. 3C. This plot was made on a single pipette whose performance is represented by the third column at each injection level

in Fig. 3A. The linear regression slopes of the five 5 μm pipettes are shown in Fig. 3D.

In summary, a feeding pressure of 0.60 MPa corresponding to a peak pulse pressure of 0.45 MPa delivered by a driving pulse of 50 ms secures a 99% ejection yield from 5 μm pipettes with mean ^{14}C -penicillin volumes between 0.17-0.5 nl depending on whether 1 or 4 pulses are delivered.

Pipettes with tip sizes 2-2.5 μm here referred to as 2 μm were tested similarly as were the 5 μm pipettes. The 2 μm pipettes would be expected to require higher pressure pulses to ascertain high reliability in ejection but occasionally low P_{e} s were tested. The T_{d} pulse duration was however not such an instance increased to 100 ms. The P_{e} s 1179, 49 and 196 kPa were tested with single pressure pulses and gave the following ^{14}C -penicillin mean amounts ($n=5$) 0.11, 0.24 and 0.85 nl. The longer pulse duration with the 2 μm pipette thus secured ejection volumes of similar magnitudes as those

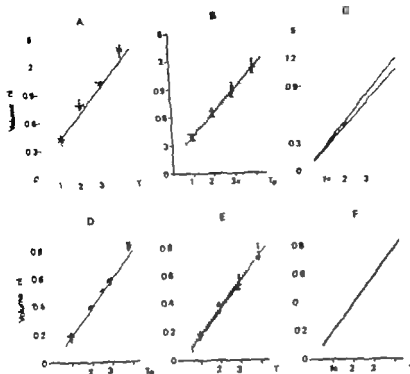


Fig. 5. Upper diagrams show linear regression slopes of ^{14}C -penicillin ejectates from a $5\ \mu\text{m}$ pipette delivered into ten slices (A) and into ten oil droplets (B) at each pressure pulse group. The injections were given alternately into the two media, with clearing pipette positions. Distribution, 95% confidence interval for mean of volumes and linear regression lines are given. C. Comparison of linear regression slopes for A and B. Lower diagrams illustrate linear regression plots of ^{14}C -penicillin ejectates from a $2\ \mu\text{m}$ pipette delivered into ten slices (D) and into ten oil droplets (E). The injections were delivered as described above. Distribution, 95% confidence interval for mean of volume and linear regression lines are given and compared, F.

obtained with half the pulse duration, from $5\ \mu\text{m}$ pipettes.

Five $5\ \mu\text{m}$ pipettes were tested in the same manner as the $5\ \mu\text{m}$ pipettes. The size of the ^{14}C -penicillin-phenylethanol was kept around $30\ \mu\text{m}$ diameter for each pipette. The spiderule size was achieved with a P of 1.5 MPa, corresponding to a P of 0.45 MPa and driving pulse of 70 ms (see method, e.g. 4A).

As shown by the histogram in Fig. 4A, the patency of the $5\ \mu\text{m}$ pipettes in 200 slices was maintained in 95% of the injections. In the pulse number group blocking occurred in 4 out of 50 single injections while in pulse number groups 4-4x no blocking occurred. The blocking incidence was thus 1% higher for the $2\ \mu\text{m}$ than for the $5\ \mu\text{m}$ pipettes tested. Data on the distribution of the individual ejectates obtained from one pipette are shown in Fig. 4C showing the 95% confidence interval for mean of

volume for the ten injections at each pressure group and the linear regression of the ^{14}C -penicillin ejectates as a function of pulse magnitude. The mean volumes and SDs are shown in Fig. 4B for the five injection series pooled together. The linear regression data of the ^{14}C -penicillin ejectates obtained from the five $5\ \mu\text{m}$ pipettes are shown in Fig. 4D. From these it may be seen that a single MPa pressure pulse of 70 ms duration on the average yields a 0.035 nl volume.

Comparing the $2\ \mu\text{m}$ and $5\ \mu\text{m}$ pipettes Figs. 3 and 4 clearly illustrate the difference in volume magnitude between the two pipette samples chosen.

In conclusion, high reliability is gained with ^{14}C -penicillin ejectates from $2\ \mu\text{m}$ pipettes with a feeding pressure of 1.5 MPa and a pulse duration of 70 ms. 2-4x pulses yield a 100% outcome, single pulse injections had a 97% yield. The mean amounts obtained with the extreme pulse number groups (1x

and $4\times$) were of the order of 0.03 and 0.15 nl respectively, corresponding to spherule diameters of 40–65 μm .

When physiological experiments with ^{14}C -penicillin are pursued, histological controls may be necessary to make. In such instances scintillation of slices may not be done. Determination of ^{14}C -penicillin ejected into oil could therefore by inference be comparable to the volume injected into a slice from one and the same pipette used with identical injection parameters.

In order to obtain information on the reliability of this approach experiments on the relation between ^{14}C -penicillin amounts injected into oil vs. slices were carried out in which the injections were done shifting the pipette from oil to slice. A 5 μm and a 2 μm pipette was tested.

The upper three diagrams of fig. 5 show the results obtained from the comparison test performed with the 5 μm pipette. The pressure parameters applied to the pipette were identical to those of the other five 5 μm pipettes investigated. A illustrates the distribution of the individual volumes obtained with $1-4\times$ pulses into the slice. B the corresponding amounts injected alternately into oil. The mean volume at each pressure pulse group in the two media are very similar, differing from each other only at the $1/10$ nl magnitude. The 95% confidence interval for mean of volumes injected into the slice (A) is somewhat broader than that of the volumes in oil (B) indicating a higher tendency to partial blockage in the slice than in the homogenous oil.

The slopes of the linear regression lines obtained from the slice and oil volumes are not identical, however, it can clearly be seen in Fig. 5C that from a practical point of view, the difference is ignorable.

The same slice vs. oil injection test done on the 2 μm pipette gave similar results. They are shown in the lower sequence of diagrams in Fig. 5. D and E illustrate the distribution of the individual volumes in slices and oil droplets, the linear regression lines now almost identical (F) and the 95% confidence interval for mean volume being illustrated. As for the 5 μm pipette, the volumes vary somewhat more in slice than in oil, but the means of each pressure level group are highly comparable, differing from each other only at the $10-30$ pl level.

Injection into oil in different pressure pulse groups either from 5 μm or from 2 μm pipettes thus provides a valuable check of the volumes injected into a slice. Consequently visual estimation of the

size of a non radioactive penicillin spherule injected into oil may serve as an estimate of a volume injected into a slice from a 5 μm or a 2 μm pipette.

DISCUSSION

Pressure microinjection is an alternative to iontophoretic and thermal methods. Early pressure microinjection methods are described by Chance & Kopac (1950). Methodological refinements have been done during the years, but already in the earliest studies considerable exactness in ejection quantities have been reported.

A particularly favourable preparation to use in studies on microinjection of substances considered crucial for nerve function was the giant axon preparation utilized by several authors shortly after the description of the injection method. Grundfest et al. (1954), Hodgkin & Keynes (1956). By combined electrolyte ejection with electrical recording from one and the same pipette Grundfest et al. improved the functional spatial accuracy of the microinjection method.

Further elaboration of the combined ejection-recording principle has later been performed by various authors and suggested as an alternative to the iontophoretic method. Krnjević & Phillips (1963), Krnjević et al. (1963), Woodward & Lindström (1977), McCaman et al. (1977), Källström & Lindström (1978). By adding the use of radioactively labelled substances, quantitative knowledge has been obtained of the effects of various substances on neuronal mechanisms. Extra- and intracellular pressure microinjection has thus been possible to make using either single or multibarrel pipette set-ups.

Several studies have been performed with pressure ejection of substances and relating the ejection quantitatively to the functional effects observed. Studies on ionic mechanisms have thus been conducted (Chang et al. 1974, Rose & Loewenstein 1976) or on transmitter physiology (Koike et al. 1977, Eisenstadt et al. 1973, Koike et al. 1974, Schwartz 1974, Tauc et al. 1974).

Methodological reports on ejection obtained with various substances, labelled radioactive or given in the literature (Zieglansberger et al. 1969, Obata et al. 1970, Clarke et al. 1973, Hoffner et al. 1971), McCaman et al. (1977).

In studies on experimentally evoked epilepsy in the neocortex, microinjection of benzylpenicillin

in addition to the use of applying penicillin soaked pledgets on the surface and depth injections have been done with iontophoretic technique (also 1971; Curtis et al 1972).

It has been argued that the iontophoretically used benzylpenicillin influences neuronal membranes or synaptic mechanisms with an excitatory result. A further desirable improvement in relating microinjection ejection to functional results, is to quantify the ejected volume and relate it to the functional response produced in a neuronal substrate of certain chaotic organization so as to minimize integrative complexity.

A first step in such an endeavour is to quantify microinjection ejection and to establish if possible the stability and volume scatter that a chosen pressure microinjection technique presents.

As blocking of the pipette is a hazard encountered in any microinjection method benzyl (¹⁴C) penicillin was used to establish the reliability of the method and scatter of ejection. In addition to a few tests performed on the relation between pulse duration and ejection volume the main interest was focused on the volumes obtained on repetition of identical pressure pulses.

By injecting into ten slices in each series knowledge was obtained on the blocking incidence of the pipettes used and of the volume scatter in the various pressure number groups.

The results show that 5 μ m pipettes perform, with the pressure parameters used, with an ejection efficiency of 99%. If pressure pulses were delivered over 4 times, 100% yield was obtained. The average ejection volume obtained from these pipettes, with one pulse was 0.13 nl, corresponding to a spherule of about 63 μ m diameter. From the 1 μ m pipettes, ejection volumes of 0.04 nl (i.e. \sim 40 μ m spherules) were obtained. The 1 μ m pipettes performed with similar high accuracy giving an average blocking incidence of only 2% if 1 pulse was used. Raising the pulse repetition to 2 \times 3 \times or 4 \times resulted in 100% yield.

The use of 1 μ m and 5 μ m tips subjected to the pressure pulses reported here thus enables ejection of volumes equal to the sizes of 1-3 neurone somata in the CA region of the guinea-pig hippocampus.

The use of visually checked injection of a substance into oil gives a good magnitude estimation of the amounts provided the viewing conditions allow high enough accuracy (Chambers & Kopac

1950). If however accuracy in quantification is to be used in functional studies the visual separation of a spherule of a particular size from that of another is hardly acceptable as distortion of the injected spherule in oil may prevent volume estimation. It is therefore advisable, in future studies, to use the oil injection method as a patency test and for magnitude estimation.

Another point to stress is that penicillin should be injected into an architecturally 'simple' nervous structure that allows single neuronal effects to be studied with the least possible circuitry complexity. It may even be possible to study the effect of microinjected penicillin on various segments of different membrane areas of single neurones. The cytoarchitectonic feature and the *in vitro* technique of the transverse hippocampal slice preparation (Skrede & Westgaard 1971) is an excellent structure for exact studies on the epileptogenic property of benzylpenicillin.

A study of the epileptogenesis of single cells in the guinea-pig hippocampal slice preparation, microinjected with penicillin may with the present method thus be conducted with accuracy.

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Effect of vagotomy and glucose administration on gastric acid secretion in the Atlantic cod *Gadus morhua*

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Cods were prepared for measurement of gastric acid secretion. Cannulae were implanted for drainage of the stomach and for separate perfusion of the stomach and intestine. Fishes intestinally perfused with diluted sea-water (33% SW) exhibited high rates of acid secretion. This "basal" secretion was abolished (96% inhibition) by bilateral vagotomy. Perfusion of the stomach with osmotic glucose (5.5%) diminished the amount of titratable acid in the gastric effluence, and induced oral ingestion of water. Only part of the decrease could be explained by neutralization by ingested sea-water. Perfusion of the intestine with 5.5% glucose, containing sea-water to raise the osmolality to that of 50% SW, depressed gastric acid secretion compared to 50% SW-perfused controls, without significantly affecting oral intake of water. I.e., administration of glucose, increasing plasma glucose to the same level as intraluminal administration, did not affect acid secretion or oral water ingestion. The study demonstrates the existence of potential intestinal mechanisms for inhibition of gastric acid secretion, and indicates that vagal activity is independent of the blood glucose level in the cod.

Key words: Fish physiology, gastric acid secretion, glucose, inhibition, vagus nerve

codfish deprived by pyloric ligation of the possibility to furnish water to the intestine become dehydrated and exhibit high rate of oral water intake and low or no gastric acid secretion. Perfusion of the intestine at a relatively high rate rapidly extinguishes drinking, while perfusion at a slower rate has a less depressant effect on the drinking rate. (The effects are the same both if the perfusion is dehydrating (pure sea-water, 100% SW) or if the perfusion permits the animal to replenish osmotically lost body water (50% or 33% SW, i.e. seawater diluted with one or two volumes of distilled water, respectively). When 50% or 33% SW is used for the intestinal perfusion, not 100% SW, gastric acid secretion is high (Holstein 1979). The mechanism driving this high "basal" secretion is unknown. Since the animals were starved at least one week before experimentation and since hypoglycemia is known to induce vagal activation and ensuing gastric hypersecretion in mammals (Collins & Himsworth 1969; Stacher *et al.* 1976)

we decided to study the effect of vagotomy and glucose administration, intraluminal and intramuscular, on codfish "basal" acid secretion.

MATERIALS AND METHODS

Animals. Codfish of both sexes were used. Before experiments they were kept in storage aquaria with recirculating, chilled (10°C) sea-water for at least one week. They were not fed.

Surgery. The animals were surgically prepared for the separate perfusion of the stomach and intestine, and for drainage of the stomach, by a method previously described in detail (Holstein 1979): the cannulae positioned were C1, C2, and C3. In addition, some animals were subjected to bilateral vagotomy. A small incision was made dorsally in the thin skin behind the posterior gill arch. Through the opening the vagus nerve, running in close contact with the duct of Cuvier, is accessible. The gastric branches were carefully lifted and cut off. Except

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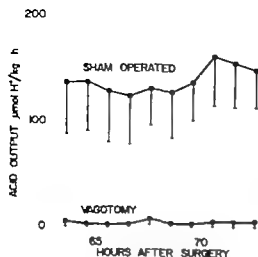


Fig. 1 Gastric acid secretion in 5 vagotomized and 5 sham-operated cods. Gastric perfusion with 33% SW. At each point the significance for the difference between the groups is $P=0.008$ (Mann-Whitney U-test). In this and the following figures means and 1 S.E. are given.

for cutting the nerves (sham-operated animals received the same treatment).

Postsurgical treatment. Immediately following recovery from anaesthesia (MS-222, Sandoz) the fishes were transferred to smaller tanks and perfusion of the intestine (33% SW) and stomach (100% or 33% SW) started. The gastric perfusion contained 100 mg/l phenol red (sodium salt, Sigma). Perfusion rate for both stomach and intestine was about 8.5 ml/h, but varied between pumps and experiments. The actual rate was recorded following each experiment. Gastric outflow was collected in 1 h fractions. In some vagotomy experiments the intestinal cannula and the pyloric ligation were omitted and perfusion was only via the stomach.

The fishes were left without further treatment for at least 20 h and were then used for the experiments during 4 postsurgical days. Final body weight was then recorded and expressed as a percentage of the presurgical body weight.

Analyses and presentation of the data

Gastric perfusion. The samples were analysed for volume, acidity and phenol red (PR) concentration as described earlier (Holstein 1979a). From the amount of PR infused and the amount of PR in the gastric effluence the recovery of perfusion fluid was calculated and outflow volume

accordingly corrected. The acid content was calculated from acidity and corrected volume and expressed as $\mu\text{mol H}^+/\text{kg h}$. In calculating this the presurgical body weight was used. In most figures acid secretion has been normalized so that the acid secretion during the hour preceding change of the experimental situation ("control h"), or mean of 5 h preceding change of the experimental situation ("control period") equalized 100%. Gastric outflow is given as a percentage of the hourly inflow volume.

Blood samples. Blood was drawn in heparinized syringes from the caudal vein and the separated plasma samples were frozen until analysed for glucose (glucose oxidase method), osmolality (Wescor 5100R Vapor Pressure Osmometer), K⁺ Na⁺ (Turner Model 510 P₇ Photometer) and Cl⁻ (Radiometer CMT 10 Chloride Ionator) concentrations.

Statistics. The Wilcoxon matched-pairs signed-rank test, the Mann-Whitney U-test, the Randomization test for independent samples or the Kruskal-Wallis one-way analysis of variance (Siegel 1956) were used as indicated in text and legends.

RESULTS

Vagotomy. Following recovery from anaesthesia gastric acid secretion in the sham-operated group gradually increased and at 5–15 h postsurgically the color of the gastric effluence turned to the acid yellow of PR. In the vagotomized group the color stayed red for the entire observation period. The secretion was assayed in 10 consecutive h as shown in Fig. 1 there was an almost complete abolition (i.e. 96% inhibition) of acid secretion following vagotomy. The mean (\pm S.E.) volume of gastric outflow during the 10 h in the vagotomized group ($104.71 \pm 0.99\%$) was not significantly different ($P>0.2$, Randomization test) from the mean volume in the sham-operated group ($106.06 \pm 0.89\%$). Neither were there any significant differences between the two groups concerning body weight or the measured plasma parameters (Table 1).

Experiments were also carried out in animals without pyloric ligation and perfused via the stomach with 33% SW-PR. Intact animals exhibited

Table 1. Some plasma parameters and body-weight (in per cent of presurgical weight) in vagotomized and sham-operated cods.

Means \pm S.E. of 5 animals each. Conc. in mM.

| | [Na ⁺] | [K ⁺] | [Cl ⁻] | mOsm/kg | % b wt |
|---------------|--------------------|-------------------|--------------------|-----------------|-----------------|
| Sham-operated | 157.6 \pm 6.6 | 3.8 \pm 0.7 | 141.6 \pm 1.9 | 298.6 \pm 6.4 | 103.6 \pm 0.9 |
| Vagotomy | 158.2 \pm 4.0 | 3.9 \pm 0.7 | 138.2 \pm 4.4 | 298.8 \pm 6.8 | 104.4 \pm 0.9 |

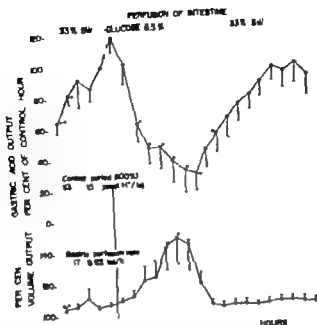


Fig. 2. Effect on gastric acid secretion and gastric volume outflow of intestinal perfusion with glucose in 8 sm eels. In this and the following figures gastric perfusion is with 100% SW-PR. In the statistical analysis (Wilcoxon test) each point was compared to the control h. $P < 0.05$, $**P < 0.01$.

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Intraluminal glucose perfusion. Upon change of the intestinal perfusion from 33% SW to 5.5% glucose, gastric acid content in the outflow decreased (Fig. 2). Following rechange to 33% SW, acid output recovered and was no longer significantly different from control four from the 5th posttreatment hour. The volume of the gastric outflow increased during glucose perfusion and then, following rechange to 33% SW, returned to the basal level. The decrease in acid output was not always associated with increased volume outflow: in one fish the volume output (5th h of glucose perfusion) was 106% of control h, and in another the only slightly increased volume (117%) occurred with an acid output amounting to 7.5% of that in the control hour.

Effect on plasma glucose and body weight. Previous experiments have shown that dehydration inhibits acid secretion and that the % b wt. can be used to measure dehydration (Holst 1979a). To study the effect on b wt. of glucose perfusion, these experiments were repeated, but the animals now killed following 6 h of intraluminal glucose. For comparison, one series in which the 33% SW perfusion continued for the same time period and another series receiving 5.5% glucose by the l.m. route (0.62 ml/h) were included. Compared to the respective control II intraluminal glucose but not l.m. glucose diminished gastric acid output (Fig. 3). The group treated with glucose intraluminal exhibited a small but significant decrease in b wt. (Table 2). Gastric volume output increased (in 4 of the 11 animals) during intraluminal glucose, not during l.m. glucose (Fig. 3, Table 3). The plasma glucose concentrations were compared to the control group; increased in the glucose-loaded groups but the difference between these later groups was not significantly different (Table 4).

Intraluminal perfusion with glucose-co-water. The glucose solution used for the intraluminal perfusion contained no salts. Since sodium chloride is

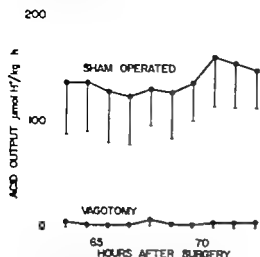


Fig. 1 Gastric acid secretion in 5 vagotomized and 5 sham-operated cods. Gastric perfusion with 33% SW. At each point the significance for the difference between the groups is $P=0.008$ (Mann-Whitney U-test). In this and the following figures means and 1 S.E. are given.

for cutting the nerves sham-operated animals received the same treatment.

Postsurgical treatment. Immediately following recovery from anaesthesia (MS 222, Sandoz) the fishes were transferred to smaller tanks and perfusion of the intestine (33% SW) and stomach (100% or 33% SW) started. The gastric perfusion contained 100 mg/l phenol red (sodium salt, Sigma). Perfusion rate for both stomach and intestine was about 8.5 ml/h, but varied between pumps and experiments. The actual rate was recorded following each experiment. Gastric outflow was collected in 1 h fractions. In some vagotomy experiments the intestinal caecula and the pyloric ligation were omitted and perfusion was only via the stomach.

The fishes were left without further treatment for at least 20 h and were then used for the experiments during 4 postsurgical days. Final body weight was then recorded and expressed as a percentage of the presurgical b.wt.

Analyses and presentation of the data

Gastric perfusion. The samples were analysed for volume acidity and phenol red (PR) concentration as described earlier (Holstein 1979a). From the amount of PR infused and the amount of PR in the gastric effluence the recovery of perfusion fluid was calculated and outflow volume

accordingly corrected. The acid content was calculated from acidity and corrected volume and expressed as $\mu\text{mol H}^+/\text{kg h}$. In calculating this the presurgical b.wt. was used. In most figures acid secretion has been normalized so that the acid secretion during the hour preceding change of the experimental situation ("control h"), or mean of 5 h preceding change of the experimental situation ("control period") equalized 100%. Gastric outflow is given as a percentage of the hourly acid volume.

Blood samples. Blood was drawn in heparinized syringes from the caudal vein and the separated plasma samples were frozen until analysed for glucose (phosphorylase method), osmolality (Wescor 5100R Vapour Pressure Osmometer), K⁺, Na⁺ (Turner Model 510 Fl Photometer) and Cl⁻ (Radiometer CMT 10 Chloride titrator) concentrations.

Statistics. The Wilcoxon matched-pairs signed test, the Mann-Whitney U test, the Randomization test for independent samples or the Kruskal-Wallis one analysis of variance (Siegel 1956) were used as indicated in text and legends.

RESULTS

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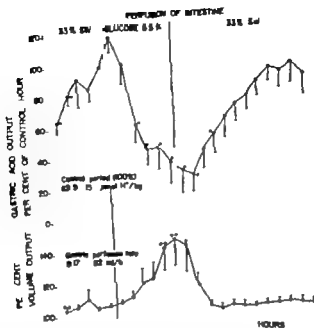


Fig. 2. Effect on gastric acid secretion and gastric volume outflow of intestinal perfusion with glucose in fish. In this and the following figures gastric perfusion is with 100% SW-PR. 1. The statistical analysis (Wilcoxon test): each point was compared to the control h. * $P < 0.05$, ** $P < 0.01$.

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Effect on plasma glucose and body weight. Previous experiments have shown that dehydration inhibits acid secretion, and that the % b wt. can be used to measure dehydration (Holstein 1979d). To study the effect on b wt. of glucose perfusion these experiments were repeated, but the animals now killed following 11 h of intraluminal glucose. For comparison, one series in which the 33% SW perfusion continued for the same time period and another series receiving 5.5% glucose by the i.m. route (0.62 ml/h) were included. Compared to the respective control h. intraluminal glucose but not i.m. glucose diminished gastric acid output (Fig. 3). The group treated with glucose intraluminal exhibited a small but significant decrease in b wt. (Table 2). Gastric volume output increased in 4 of the 6 animals during intraluminal glucose but not during i.m. glucose (Fig. 3, Table 3). The plasma glucose concentrations were compared to the control group: increased in the glucose-loaded groups but the difference between these later groups was not significantly different (Table 4).

Intraluminal perfusion with glucose isoelectric.

The glucose solution used for the intraluminal perfusion contained no salts. Since sodium chloride is

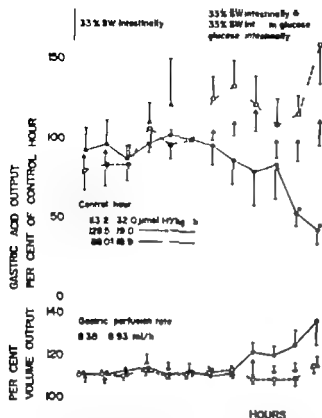


Fig. 3. Effect on gastric acid secretion and gastric volume outflow of intramuscular or intraluminal administration of 5.5% glucose. $P < 0.05$ for the difference from the control h in the respective series using the Wilcoxon test. Further analyses regarding the volume output are presented in Table 3. $n = 6$ in each group.

Table 2. Body weight in per cent of presurgical weight of glucose-loaded coals (cf. Fig. 3)

| Group | Treatment | % b wt S.E. | Statistics |
|-------|----------------------|----------------|--------------------|
| A | Control | 102.3 ± 0.5 | A vs B NS |
| B | Glucose i.m. | 104.1 ± 0.7 | A vs C $P = 0.004$ |
| C | Glucose i. huminally | 98.7 ± 0.8 | B vs C $P = 0.007$ |

Analysis of variance demonstrated a significant ($P < 0.01$) difference among the groups. The two-sample comparison employed the Mann-Whitney U test.

Table 3. Comparison of gastric mean volume outflow before and during treatment with glucose (cf. Fig. 3)

| Group | Treatment | Volume outflow (%) Gross mean ± S.E. | Significance |
|-------|---------------------------------|---|--------------|
| A | Control 6 h before treatment | 111.23 ± 0.80 | NS |
| | Control 6 h during treatment | 111.66 ± 1.07 | |
| B | 6 h before i.m. glucose | 112.45 ± 1.21 | NS |
| | 6 h during i.m. glucose | 114.1 ± 1.46 | |
| C | 6 h before intraluminal glucose | 112.31 ± 0.77 | $P < 0.01$ |
| | 6 h during intraluminal glucose | 115.53 ± 0.77 | |

Randomization test $n = 36$ in each group (6 animals each)

essential for the resorption of water from the lumen. The lack of ions might be responsible for the excessive loss of weight. The experiment was therefore modified and the intestine perfused with a mixture of 5.5% glucose in distilled water and 5.5% glucose in sea water. The proportions were such that the osmolality of the mixture equalled that of 50% SW and then contained 17% SW. Fishes perfused 50% SW served as controls. Perfusion of the intestine with glucose-SW inhibited acid secretion (Fig. 4) without significantly affecting volume output (Fig. 4, Table 5) volumes surpassing basal occurred only in one of the fishes. Again the glucose-containing perfusion caused a decrease in b wt (Table 6) but the final b wt was less than 100% only in 3 of the 6 glucose treated animals.

Correction for neutralization. Gastric volume output represents infused volume (i ml) and, at basal state, s ml corresponding to gastric secretion and water lost to the gastric lumen by osmosis (Holstein 1979b). The basal output $((i+s)/i) \times 100$ was 105–115%.

During intestinal perfusion with glucose the volume output often increases. Suppose a fish is perfused during intestinal acidification (Holstein 1979b), then this is due to drinking of d ml sea water. The volume output is then $((i+s+d)/i) \times 100$ expressed in the usual way as a percentage of the infused volume.

Table 4. Plasma glucose concentration in glucose loaded cods (cf Fig 3)

| Treatment | mg/100 ml \pm S.E. | Statistics |
|--|----------------------|------------------|
| Control | 50.14 \pm 3.13 | A vs B $P=0.002$ |
| 5.5% glucose i.m. (34 mg/h \times 6 h) | 264.41 \pm 39.18 | A vs C $P=0.002$ |
| 5.5% glucose i.m. (450 mg/h \times 6 h) | 148.22 \pm 52.41 | B vs C NS |

Analyses of variance demonstrated significant ($P < 0.01$) difference among the groups. Two-sample comparisons employed Mann-Whitney U-test.

due to its content of bicarbonate the ingested seawater neutralizes part of the secreted acid, leading to underestimation of acid secretion. A correction curve was constructed by mixing constant volume samples (1 ml) from a pool of gastric effluence with increasing volumes of sea-water (d ml). The acid content was calculated from titrated acidity and total volume (i + d') and plotted against (i + d) / 100, i.e. against "per cent volume output". The amount of acid lost by neutralization in the experimental samples was estimated from the correction curve by reading at the percentage volume output following subtraction of (d') \times 100, i.e. the percentage increase above infused volume. The obtained value recalculated per kg b.wt. was added to the acid output measured as described in the methodology. Although this procedure elevated the secretory curves, the effect was slight and only slightly affected the statistical analyses. In Fig. 4, all points marked with asterisks were still significantly different ($P < 0.05$) from control b though sometimes at a lower level than before the correction. In Fig. 3 however the acid output during the final h, though depressed was no longer significantly different from the control b. In Fig. 4 where the increase in volume was least the correction did not affect the statistical analyses.

DISCUSSION

Gastric acid secretion in 33% SW-intestinally perfused codfish is high. This work demonstrates that an intact vagal supply is obligatory for this "basal" secretion. Previous results have indicated that cholinerger mechanism is involved, carbachol stimulates (Holstein 1976, 1977) and atropine and hexamethonium inhibits (Holstein 1979a) codfish gastric secretion. Vagal involvement in fish digestive

processes has previously been suggested by Russian authors, as cited by Kapoor et al. (1975).

The almost complete blockade of acid secretion following section of the vagi must not be interpreted to indicate that the "basal" secretion is driven solely or even partly by direct vagal innervation. The same results would be expected if vagal activity regulates the release of a stimulating hormone. In mammals gastric acid secretion is regulated by direct vagal routes and by vagus-controlled release of gastrin. albeit the relative magnitude varies between species (for references, see Rayford & Thompson 1977). It is also possible that the sen-

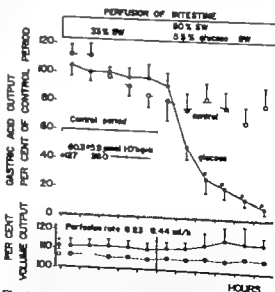


Fig. 4. Gastric acid secretion and gastric volume outflow during perfusion of the intestine with 50% SW ($n=5$) and mixture of glucose and SW isotonic to 50% SW ($n=6$). For acid secretion comparisons were between the series. $P < 0.004$ (Mann-Whitney U-test). For volume outflow comparisons were with the respective control periods, indicated to the left (see also Table 5).

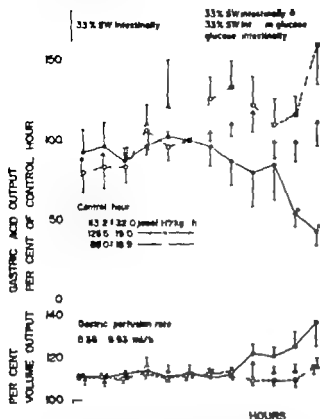


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Randomization test: $n = 36$ in each group (6 animals each).

essential for the resorption of water from the lumen. The lack of ions might be responsible for the increased loss of weight. The experiment was then modified and the intestine perfused with a mix of 5.5% glucose in distilled water and 9.5% glucose in sea water. The proportions were such that the osmolality of the mixture equalled that of 9.5% glucose and then contained 17% SW. It has proved that 50% SW served as controls. Perfusion of the intestine with glucose SW inhibited acid secretion (Table 4) without significantly affecting volume output (Fig. 4, Table 5). Volumes surpassing basal occurred only in one of the fishes. Again the glucose-containing perfusion caused a decrease in body weight (Table 6) but the final body weight was less than 10% only in 3 of the 6 glucose-treated animals.

Correction for neutralization. Gastric volume output represents infused volume (i ml) and m ml basal state s ml corresponding to gastric secret and water lost to the gastric lumen by osmosis (Holstein 1979b). The basal output ($(i+s)/i \times 100$) was 105–115%.

During intestinal perfusion with glucose the volume output often increases. Suppose as is the case during intestinal acidification (Holstein 1979b) that this is due to drinking of d ml sea-water. The volume output is then $(i+s+d)/i \times 100$ expressed in the usual way as a percentage of the infused volume.

ance (as indicated by the decrease in b wt.) or to glucose-dependent activation of a nervous or hormonal reflex mechanism.

Dehydration per se is known to inhibit the gastric secretion (Holstein 1979a). Compared to control a decreased mean b wt. was observed in the case-perfused series but the decrease—though numerically significant—was slight in fishes perfused with 5.5% glucose containing sea-water (Table 6 Fig. 4). In this series, marked inhibition was observed in all individuals despite the fact that 3 of 6 animals had a final b wt. exceeding the pre-original weight. Therefore it seems justified to exclude dehydration as the main cause of the inhibition and we suggest that there exists in the codfish glucose-sensitive, intestinal mechanism for control of gastric acid secretion. Previously a similar mechanism activated by intestinal acidification has been demonstrated (Holstein 1979b).

In mammals, intestinal stimulation of hyperosmotic glucose inhibits gastric acid secretion (Christiansen & Hendel 1974, Christiansen et al. 1976, Ward et al. 1977). GIP, gastric inhibitory polypeptide, is a potent acid secretory antagonist (Pederson & Brown 1977) and is released by luminal glucose perfusion (Jarrimer et al. 1978, Sirinek et al. 1977, Sirinek et al. 1978, Thomas et al. 1977). GIP may prove to be a enterogastrone responsible for glucose-induced inhibition of mammalian acid secretion (Brown et al. 1974, Pederson et al. 1978). In fish however neither the existence of GIP nor its effect on acid secretion is known.

The attenuation (low rate perfusion) or extinguishing (high rate perfusion) of drinking in dehydrated codfish during intestinal perfusion with pure or variously diluted sea-water was interpreted to indicate the presence of intestinal volume receptors regulating oral intake of water (Holstein 1979a). The effects are similar to those observed in monkey (Maddison et al. 1977), but differs in the respect, not in this species duodenal infusion of saline was ineffective to stop gastric sham drinking. This may indicate different mechanisms in eliciting preabsorptive satiety. For further discussion of this concept is referred to the reviews by Andersson (1978) and Zimmerman (1977). A recent report (Holstein 1979b) shows that drinking in the codfish occurs during acidic intestinal perfusion (high rate), and the present one indicates that this is probably the case also using glucose perfusion (high rate). The effect may be explained by intrinsic drink-stimulating properties

of the infused solutions, or by derepression of the drink reflex. It was thought that drinking might be due to subthreshold concentration in the lumen of some sea-water component, and the inclusion of 17% SW in the glucose perfusion in diminishing the volume-increase seemed to corroborate this concept. However the acidic perfusion (Holstein 1979b) contained 22% SW and yet induced a prominent but transient volume increase. On the very extreme end perfusion of the intestine for 6 h with distilled water induced a slight (not significant) volume increase without causing any water deficit (unpublished observations in 8 animals). Clearly more work is needed before the drinking behaviour during intestinal perfusion can be satisfactorily explained. However there still seems to be room for the speculation that ingestion of alkaline sea-water is utilized as an additional means to lower the gastric acidity.

This work was made possible by grants from Aarne Ahrensbergs fond for vetenskapliga syften, S. Andersson, Harald Jeansson, Sjöfiske och Harald och Greta Jeansson Stiftelse and from Långsmånka Institutionen. We wish to thank Mrs Laga-Maj Orborn for expert technical assistance and Mr Ingemar Halmen for supply of fish. We are indebted to the Captain and his staff onboard Tor Bränsman for bringing water from the North Sea to the Institute's aquarium.

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Table 5. Comparison of gastric mean volume outflow before and during intestinal perfusion with 40°C SW (5 animals) and before and during perfusion with sea-water containing 5.5°C glucose (6 animals) (cf. Fig. 4). n = number of fish in the gross mean.

| Treatment | n | Volume outflow (ml) Gross mean \pm S.E. | Significance (Randomization test) |
|---------------------------------|-----|--|--------------------------------------|
| 5 h before 40°C SW perfusion | 5 | 106.60 \pm 0.3 | NS |
| 6 h during 40°C SW perfusion | 30 | 106.70 \pm 0.31 | |
| 5 h before glucose-SW perfusion | 30 | 111.77 \pm 1.26 | NS |
| 6 h during glucose-SW perfusion | 36 | 114.38 \pm 1.1 | |

sensitivity of the secretory cell for other secretagogues is decreased following vagotomy, as has been observed in cats and dogs (Andersson & Grossman 1965; Emås & Grossman 1967; Olbe 1963; Svensson & Emås 1977).

Compared to freshly trawled cod (Larsson et al. 1976), the plasma glucose level in the present starved control animals appears normal. Intravenous injection of glucose rendered highly hyperglycemic animals, but did not affect the secretion of gastric acid. This contrasts to findings in mammals, where artificially increased (by parenteral administration) blood glucose levels inhibit both basal and postprandial secretion (Dotevall & Muren 1961; Kemp et al. 1968; Moore 1973; Solomon & Spiro 1959; Stacher et al. 1976), though concerning pentagastrin-induced secretion the results are conflicting (Holst & Christiansen 1975; Markiewicz & Lukin 1976). The mammalian lateral hypothalamus contains glucose-sensitive chemoreceptors (Colm-Jones & Himsworth 1970); the inhibition probably being mediated via these structures. Our results seem irreconcilable with the existence of a similar mechanism in the cod. Also in the chicken, the response of acid secretion to changes in metabolizable glucose levels deviates from that in the mammal (Burhol & Hirschowitz 1971; Ruoff & Sewing 1971). In another fish, the blood glucose level has been shown to regulate appetite (Kuzmina 1967); the present results do not exclude such a mechanism in the cod.

Perfusion of the intestine with isosmotic glucose elevated the plasma glucose to a level comparable to that following intravenous administration, but in contrast to the i.v. route intraluminal glucose depressed the acid output. The interpretation of this result is complicated by i) the increased volume output and ii) the decreased b.w.t. accompanying glucose per-

fusion. The increased volume may reflect a greater volume flow (active secretion or passive flux along the tissue-to-lumen osmotic gradient) from the mucosa or ingestion of water. A similar increase in gastric volume outflow was observed during acidic perfusion of the intestine; this increase was due to oral ingestion of water (Holstein 1978b). By analogy, we consider the "drinking alternative" the most likely. If this is correct, the neutralization of secreted acid by sea-water has to be considered. A correction was applied and found to have only minor influence on the results. Moreover, during intestinal glucose perfusion, the decrease in acid output was not always accompanied by increased volume output. When sea-water was included in the glucose perfusion (total osmolality equal to 40°C SW), the volume increase was insignificant, but acid output still declined. Hence, it is concluded that the major part of the observed decrease in acid output represents a glucose-induced inhibition, and the neutralization may contribute slightly. It is also concluded that the inhibition is not related to the osmolality of the luminal content, though the molar tendency of 1 M glucose shows that the inhibitor emerges from the intestine. However, the inhibitor may be due either to interference with the water

Table 6. Body weight in percent of pre-weighed weight following 6 h of intestinal perfusion with 40°C SW or in sea-water containing 5.5°C glucose solution isomestic to 40°C SW (cf. Fig. 4).

| Group | Weight \pm S.E. | P (Mann-Whitney U-test) |
|-------------------------------|----------------------|-------------------------------|
| 40°C SW perfusion | 100.8 \pm 0.9 | 0.018 |
| 5.5°C glucose SW perfusion | 100.0 \pm 0.6 | |

balance (as indicated by the decrease in b wt.) or to glucose-dependent activation of a nervous or hormonal reflex mechanism.

Dehydration *per se* is known to inhibit the basal secretion (Holstein 1979a). Compared to control, decreased mean b wt. was observed in the glucose-perfused series, but the decrease—though numerically significant—was slight in fishes perfused with 5.5% glucose containing sea-water (Table 6, Fig. 4). In this series, marked inhibition was observed in all individuals despite the fact that 3 of the 11 animals had a final b wt. exceeding the pre-surgical weight. Therefore, it seems justified to exclude dehydration as the main cause of the inhibition and we suggest that there exists in the codfish a glucose-sensitive intestinal mechanism for control of gastric acid secretion. Previously a similar mechanism activated by intestinal acidification has been demonstrated (Holstein 1979b).

In mammals, intestinal instillation of hypertonic glucose inhibits gastric acid secretion (Christiansen & Hendel 1974; Christiansen *et al.* 1976; Ward *et al.* 1969). GIP (gastric inhibitory polypeptide) is a potent acid secretory antagonist (Pederson & Brown 1977) and is released by luminal glucose perfusion (Lammner *et al.* 1978; Smock *et al.* 1977; Srinick *et al.* 1978; Thomas *et al.* 1977). GIP may prove to be the enterogastric responsible for glucose-induced inhibition of mammalian acid secretion (Brown *et al.* 1975; Pederson *et al.* 1978). In fish however neither the existence of GIP nor its effect on acid secretion is known.

The attenuation (low rate perfusion) or extinguishing (high rate perfusion) of drinking in dehydrated codfish during intestinal perfusion with pure or variously diluted sea-water was interpreted to indicate the presence of intestinal volume receptors regulating oral intake of water (Holstein 1979a). The effects are similar to those observed in monkey (Maddison *et al.* 1977) but differs in the respect that in this species duodenal infusion of saline was ineffective to stop gastric sham drinking. This may indicate different mechanisms in eliciting preabsorptive satiety. For further discussion of this concept is referred to the reviews by Andersson (1978) and Christianson (1972). A recent report (Holstein 1979b) shows that drinking in the codfish occurs during acidic intestinal perfusion (high rate) and the present one indicates that this is probably the case also during glucose perfusion (high rate). The effect may be explained by intense drink-stimulating properties

of the infused solutions or by derepression of the drink reflex. It was thought that drinking might be due to subthreshold concentration in the lumen of some sea-water component, and the inclusion of 17% SW in the glucose perfusion, in diminishing the volume-increase seemed to corroborate this concept. However the acidic perfusion (Holstein 1979b) contained 22% SW and yet induced a prominent but transient volume increase. On the very extreme end perfusion of the intestine for 6 h with distilled water induced a slight (not significant) volume increase without causing any water deficit (unpublished observations in 8 animals). Clearly more work is needed before the drinking behaviour during intestinal perfusion can be satisfactorily explained. However there still seems to be room for the speculation, that ingestion of alkaline sea-water is utilized as an additional means to lower the gastric acidity.

This work was made possible by grants from Anna Ahrensbergs fond för vetenskapliga in ö. Andersnäs, Harald Jeansson Stiftelse och Harald och Greta Jeansson Stiftelse and from Lämningskassan i Göteborg. We wish to thank Mrs Inga-Lilja Örtengren for expert technical assistance and Mr Ingemar Hakansson for supply of fish. We are indebted to the Captain and his staff onboard Tor Britanica for bringing water from the North Sea to the institute aquarium.

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Table 5 Comparison of gastric mean volume outflow before and during intestinal perfusion with 50% SW (5 animals) and before and during perfusion with sea water-containing 5.5% glucose (6 animals) (cf Fig 4). n = number of h in the gross mean

| Treatment | n | Volume outflow (%) Gross mean \pm S.E. | Significance (Randomization test) |
|---------------------------------|-----|---|--------------------------------------|
| 5 h before 50% SW perfusion | 25 | 106.60 \pm 0.37 | NR |
| 6 h during 50% SW perfusion | 30 | 106.70 \pm 0.31 | |
| 5 h before glucose-SW perfusion | 30 | 111.72 \pm 1.6 | NS |
| 6 h during glucose SW perfusion | 36 | 114.38 \pm 2.12 | |

sitivity of the secretory cell for other secretagogues is decreased following vagotomy as has been observed in cats and dogs (Andersson & Grossman 1965; Emås & Grossman 1967; Olbe 1963; Svensson & Emås 1977).

Compared to freshly trawled cod (Larsson et al 1976) the plasma glucose level in the present starved control animals appears normal. Intravenous injection of glucose rendered highly hyperglycemic animals as the glucose level was increased about 4 times but did not affect the secretion of gastric acid. This contrasts to findings in mammals where artificially increased (by parenteral administration) blood glucose levels inhibit both basal and postprandial secretion (Dotevall & Muren 1961; Kemp et al 1968; Moore 1973; Solomon & Spiro 1959; Stacher et al 1976) though concerning pentagastric induced secretion the results are conflicting (Holst & Christiansen 1975; Markiewicz & Lukin 1976). The mammalian lateral hypothalamus contains glucose-sensitive chemoreceptors (Collin Jones & Humsforth 1970) the inhibition probably being mediated via these structures. Our results seem irreconcilable with the existence of a similar mechanism in the cod. Also in the chicken the response of acid secretion to changes in metabolizable glucose levels deviates from that in the mammal (Burhol & Hirschowitz 1971; Ruoff & Sewing 1972). In another fish the blood glucose level has been shown to regulate appetite (Kuzmina 1967) the present results do not exclude such a mechanism in the cod.

Perfusion of the intestine with isosmotic glucose elevated the plasma glucose to a level comparable to that following intravenous administration but in contrast to the i.m. route intraluminal glucose depressed the acid output. The interpretation of this result is complicated by i) the increased volume output and ii) the decreased b.wt. accompanying glucose per-

fusion. The increased volume may reflect a greater volume flow (active secretion or passive flux along the tissue to lumen osmotic gradient) from the mucosa or ingestion of water. A similar increase in gastric volume outflow was observed during acidic perfusion of the intestine; this increase was due to oral ingestion of water (Holstein 1979b). By analogy we consider the "drinking alternative" the most likely. If this is correct the neutralization of secreted acid by sea water has to be considered. A correction was applied and found to have only minor influence on the results. Moreover during intestinal glucose perfusion the decrease in acid output was not always accompanied by increased volume output. When sea-water was included in the glucose perfusion (total osmolality equal to 50% SW) the volume increase was insignificant but acid output still declined. Hence it is concluded that the major part of the observed decrease in acid output represents a glucose-induced inhibition and the neutralization may contribute slightly. It is also concluded that the inhibition is not related to the osmolality of the luminal content though the inefficiency of 1 M glucose shows that the inhibitor emerges from the intestine. However the inhibitor may be due either to interference with the water

Table 6 Body weight in per cent of preoperative weight following 6 h of intestinal perfusion with 50% SW or a sea-water containing 5.5% glucose solution isosmotic to 50% SW (cf Fig 4).

| Group | % b.wt. \pm S.E. | P (Mann-Whitney U-test) |
|------------------------------|-----------------------|-------------------------------|
| 50% SW perfusion | 102.8 \pm 0.9 | 0.018 |
| 5.5% glucose SW perfusion | 100.0 \pm 0.8 | |

alence (as indicated by the decrease in b.wt.) or to glucose-dependent activation of a nervous or hormonal reflex mechanism.

Dehydration per se is known to inhibit the "basal" secretion (Holstein 1979a). Compared in controls a decreased mean II wt. was observed in the glucose-perfused series, but the decrease—though numerically significant—was slight in fishes perfused with 5.5% glucose containing sea-water (Table 6 Fig. 4). In this series, marked inhibition was observed in all individuals despite the fact that 3 of the 6 animals had a final b.wt. exceeding the pre-surgical weight. Therefore it seems justified to exclude dehydration as the main cause of the inhibition and we suggest that there exists in the codfish a glucose-sensory intestinal mechanism for control of gastric acid secretion. Previously a similar mechanism activated by intestinal acidification has been demonstrated (Holstein 1979b).

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This work was made possible by grants from Astra Abresbergs fond för vetenskapliga st. Endast Harald Jeansson Softefse och Harald och Grete Jeansson Solfelse and from Läppnäska kulturorden. We wish to thank Mrs Inga-Bell Örteng for expert technical assistance and Mr Ingemar Håkansson for supply of fish. We are indebted to the Captain and his staff onboard *Tor Britzenski* for bringing water from the North Sea to the institute's aquarium.

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Vasomotor effects of facial nerve stimulation on cholinergic vasodilation in the eye

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The effect of facial nerve stimulation on ocular blood flow was studied in rabbits. The intracranial part of the facial nerve was stimulated electrically and the regional blood flow was measured with labelled microspheres. Effects on the intraocular pressure were determined in separate series of experiments. Stimulation increased choroidal blood flow by about 200%. The blood flow of the iris and the ciliary processes also increased. The blood flow of the eyelids and the sclerating membrane increased by approximately 1000% and the blood flow of Harder's gland increased by about 200%. The blood flow of the tongue and the submandibular gland also increased. The increase in the arterial blood flow could not be abolished by a cholinergic or ganglionic blockade. Ganglionic blockade abolished most of the increase in the blood flow of the eyelids, sclerating membrane and Harder's gland, the cholinergic blockade seemed less effective. The intraocular pressure increased with mean value of 6 mmHg during optimal (20-30 Hz) stimulation. The increase could not be prevented by cholinergic blockade. Much of the increase in arterial blood flow seemed to be caused by stimulation of nonconventional nerves. It is suggested that these nerves may be peptidergic using VIP as transmitter and lacking a hexamethonium sensitive yacine between the site of stimulation and the eye. Their nature—efferent or afferent—remains unknown. A great part of the increase in blood flow of the eyelids, sclerating membrane and Harder's gland seemed to be due to other mechanisms involving nerves with a peripheral synapse.

Key words. Facial nerve, blood flow, intraocular pressure, eyes, eyelids, sclerating membrane, Harder's gland, submandibular gland, tongue.

Very little is known about the influence of the facial nerve on ocular blood flow and aqueous humor dynamics. According to Ruskell (1965, 1970a) non-adrenergic nerves from the sphenopalatine ganglion of facial nerve origin distribute to the eye in the rabbit, monkey and man. At least in the monkey a considerable part of these nerves seems to terminate in the wall of arterioles in the choroid (Ruskell 1971) which indicates that they may influence ocular blood flow. Intraocular pressure (IOP) has been reported to increase upon stimulation of the greater superficial petrosal nerve in cats and rabbits (Gloster 1961) and interruption of the facial nerve pathway seems to cause a reduction in IOP (Ruskell 1970b).

The facial nerve has been regarded as containing

vasomotor nerves for the lacrimal and nasal glands, the sublingual and submandibular glands and the tongue. Erci & Uvula (1951) showed that stimulation of the chorda-tympani nerve in cats induces vasodilation in the tongue probably due to efferent fibres. Hellekaas (1977) by stimulating the chorda-tympani proper nerve in rats was able to demonstrate a significant decrease in the constant flow perfusion pressure of the tongue which was due to stimulation of efferent nerve fibres according to classical criteria. Eccles & Wallis (1974) also demonstrated vasodilation in the tongue with a plethysmographic technique during stimulation of the lingual nerve in cats.

Stimulation of the chorda-tympani nerve in cat was demonstrated as early as 1858 by Bernard to

induce vasodilation in the submandibular gland. This effect was analyzed recently by Gautvik (1970a, b, c). The vasodilation of the tongue and the submandibular gland induced by nerve stimulation was very atropine resistant (Erici & Uvnäs 1952; Gautvik 1970c).

The purpose of the present investigation was to study the effect of stimulation of the facial nerve on the blood flow in the eye and the intraocular pressure. Some other organs have also been studied primarily in order to check the selectivity of the stimulation. The possibility that co-stimulation of the trigeminal nerve was involved in the effects observed was also investigated.

MATERIALS AND METHODS

Albino rabbits of either sex weighing 1.7–3.0 kg were used. The animals were anesthetized with i.v. pentobarbital sodium 40–50 mg/kg b.w. and were artificially ventilated. The P_{CO_2} , P and pH of the arterial blood were checked and the ventilation adjusted so that normal acid-base levels resulted. Both femoral arteries were cannulated, one for blood sampling and the other for blood pressure measurement. The animals were thoracotomized and the left heart ventricle was cannulated. All cannulae were heparinized. Whenever needed a mixture of Macrodex (Pharmacia, Uppsala) and isotonic saline (1:1) was infused intravenously to increase the blood pressure. The amount of this mixture did not exceed 40 ml. The animals were kept warm with a heating pad.

Intracranial stimulation of the facial nerve

The facial nerve was stimulated intracranially at the internal acoustic pore with stereotaxic technique. The skull of the animal was firmly fixed in a stereotaxic frame. A small hole was bored in the skull at the sagittal suture so that the electrode could be inserted obliquely through the sagittal suture or 0.5 mm laterally at the side to be stimulated. The electrode was inserted 6.5–8.5 mm posterior to the bregma at a 16° lateral angle in the vertical axis, 21–24 mm obliquely inferior—calculated from the upper surface of the skull bone at the bored hole. The coordinates were calculated for the rabbit head which was in a slightly backward leaning position (10–15°) in the stereotaxic instrument. The electrode was left in the position where good lacrimation could be obtained. In most of the experiments lacrimation was combined with salivation. Stimulation was carried out with unidirectional square wave pulses through a bipolar electrode. The distance between the poles of the electrode was 1 mm. Stimulation parameters were: pulse intensity 6–14 V, duration 1 ms, and frequency ranging from 5 to 60 Hz, mostly being 40–50 Hz. The nerve was stimulated for 4–5 min. After the experiment the position of the electrode was checked. Four experiments were performed with facial nerve stimulation after intracranial transection of the trigeminal nerve made 5–8 mm anterior to the place where the nerve enters the mid-

Table 1. Effect of facial nerve stimulation on blood flow.

Indicates $P < 0.01$ and $P < 0.001$, $n = 18$. Differences were calculated from paired data.

| Tissue | Stimulated side (mg/min) | Control side (mg/min) | Difference (mg/min) |
|-------------------|--------------------------|-----------------------|---------------------|
| Choroid | 2.413 ± 237 | 900 ± 106 | 1.513 ± 1 |
| Iris | 157 ± 19 | 90 ± 15 | 67 ± 18 |
| Ciliary processes | 106 ± 10 | 81 ± 11 | 25 ± 8 |

dle cerebral fossa. The skull was widely opened and part of the hemisphere had to be removed before transection.

Determination of regional blood flow

Regional blood flow was determined with 15μ m radioactively labelled microspheres (3M Company) according to the reference flow method (see Alm & Bill 1972). The microspheres were injected into the left heart ventricle. Each animal received 2–3 separate injections with different labelled microspheres. It was thus possible to determine the blood flow at two to three different times during the experiment, e.g. during stimulation before pharmacologic blockade, after pharmacologic blockade and after pharmacologic blockade during stimulation. The method is described in more detail elsewhere (Stjernschantz & Bill 1976). The tissues studied were the choroid, ciliary processes scraped off the back side of the iris, the iris, the eyelids, the nictitating membrane, Harderian gland, the submandibular and parotid glands, the tongue, the masseter muscle and the facial skin. The first 100 μ l blood samples were assayed in a three-channel gamma spectrometer. The blood flow in the choroid, ciliary processes and the iris is presented as the blood flow of the whole organ (mg/min) while that of the other organs is presented as the blood flow per g tissue (mg/min/g).

Pressure measurements

The intraocular and blood pressures were measured with pressure transducers. The eyes were cannulated with a needle gun and a slow infusion (0.5 μ l/min) of an electrolyte solution (Bárdány 1964) was used to keep the cannula open. This only slightly affected the IOP. After cessation of stimulation of 3–5 min the IOP was given time to return to the baseline and stabilize for 5–10 min before the next stimulation. The animals used for IOP measurements did not receive any sphere injections and consequently were not thoracotomized.

Pharmacologic blockade

All the animals underwent neuromuscular blockade with tubocurarine 0.5–1 mg/kg b.w.t. administered in order to avoid movement of the eyelids during stimulation. Cholinergic (muscarinic) blockade was induced by atropine 2.5–10 mg/kg b.w.t. administered. Ganglion blockade was carried out with hexamethonium 50–100 mg/kg b.w.t. i.v. administered.

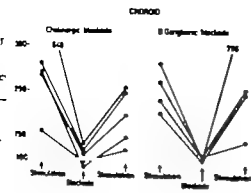


Fig 1

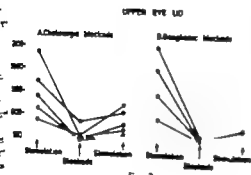


Fig 2

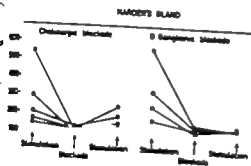


Fig 3

RESULTS

Stimulation caused lacrimation and in most of the experiments salivation. A white lipid substance was also secreted probably from the eyelids. Lacrimation and salivation were totally prevented by cholinergic and ganglionic blockade but transection of the fifth cranial nerve prior to stimulation of the facial nerve did not affect the responses. Stimulation had no appreciable effect on the pupil size and caused little change in the blood pressure.

The effect of facial nerve stimulation on the uveal blood flow is presented in Table 1. The mean increase in the choroidal blood flow was $1 \pm 38\%$

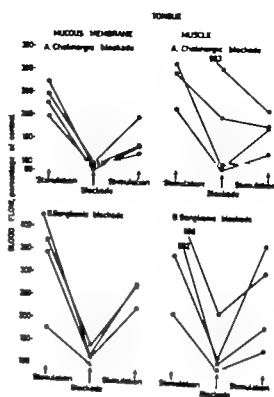


Fig 4

Figs. 1-4 Effect of pharmacologic blockade on the vasomotor effect of facial nerve stimulation. The blood flow on the stimulated side was expressed as percentage of that on the control.

($P < 0.001$ paired data). The increase in blood flow in the iris and ciliary processes was less but statistically significant at the $P < 0.01$ level. Fig. 1 shows that cholinergic blockade with biperiden did not adequately abolish the increase in the choroidal blood flow during stimulation. Ganglionic blockade with hexamethonium was in a similar way ineffective (Fig. 1B). Transection of the fifth cranial nerve 60-90 min before stimulation of the facial nerve did not prevent the vasomotor response. The blood flow in the iris and the ciliary responded in a similar way to that of the choroid after cholinergic and ganglionic blockade and after transection of the fifth cranial nerve.

Table 2 Effect of facial nerve stimulation on the blood flow in tissues innervated by the facial nerve indicates $P \leq 0.05$, $P \leq 0.005$ and $P \leq 0.001$. The mean difference was calculated using paired data

| Tissue | n | Stimulated side (mg/min/g) | Control side (mg/min/g) | Difference (mg/min/g) |
|-----------------------|----|----------------------------|-------------------------|-----------------------|
| Upper eyelid | 18 | 1795 ± 199 | 158 ± 33 | 1136 ± 182 |
| Lower eyelid | 18 | 1174 ± 70 | 153 ± 33 | 1021 ± 191 |
| Nictitating membrane | 18 | 986 ± 164 | 100 ± 17 | $887 \pm 149^*$ |
| Harder's gland | 18 | 1017 ± 193 | 380 ± 91 | 637 ± 176 |
| Submandibular gland | 10 | 736 ± 112 | 322 ± 70 | 414 ± 146 |
| Tongue (muc. membr.) | 17 | 1578 ± 353 | 48 ± 6 | 1097 ± 316 |
| Tongue (muscle layer) | 17 | 217 ± 61 | 53 ± 7 | 164 ± 62 |

The blood flow of the upper and lower eyelids and of the nictitating membrane increased almost ten times (Table 2). The blood flow of the mucous membrane and the muscle layer of the tongue also strongly increased and the same was true for the blood flow of the submandibular gland in the 10 experiments in which there was a clear salivation (Table 2). Contrary to this, the blood flow of the parotid gland, masseter muscle and the facial skin showed only minute changes during stimulation.

Cholinergic blockade partly inhibited the increase in the blood flow of the upper eyelid and of Harder's gland (Fig. 2A and 3A) but ganglionic blockade with hexamethonium inhibited most of the increase in the blood flow of these structures during stimulation (Fig. 2B and 3B). Transection of the fifth cranial nerve 60–90 min before stimulation of the facial nerve did not change the response to stimulation. The blood flow of the lower eyelid and the nictitating membrane responded in a similar way to that of the upper eyelid and Harder's gland after cholinergic

and ganglionic blockade and after transection of the fifth cranial nerve. In the tongue stimulation the facial nerve caused dilation even after cholinergic and ganglionic blockade (Fig. 4). The arterial blood pressure during stimulation after cholinergic blockade was 74 ± 6 mmHg and the corresponding value after ganglionic blockade was 70 ± 6 mmHg.

Stimulation of the facial nerve caused a rise in IOP of 5.9 ± 1.3 mmHg at 20–30 Hz; higher frequencies did not further increase the pressure (Fig. 5). The IOP before the injection was 14–20 mmHg. The increase in IOP was essentially unilateral; there was a slight change in IOP on the contralateral side only when stimulation affected the blood pressure. It rapidly increased upon stimulation. The maximal value reached within 10–20 s was usually followed by a slight fall in IOP. After stimulation the return of IOP to the prestimulation level generally took 30–60 s. A typical result is shown in Fig. 6. Cholinergic blockade with biperiden did not prevent the increase in IOP.

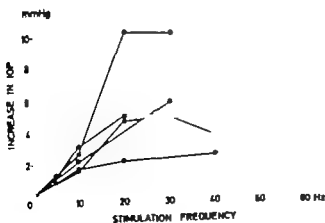


Fig. 5 The relationship between stimulation frequency and a change in the IOP during facial nerve stimulation.

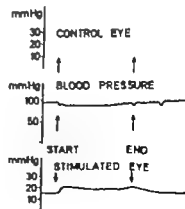


Fig. 6 IOP and blood pressure measurement in a typical experiment. Note the abrupt and somewhat transient increase in IOP upon stimulation. Stimulation period is indicated.

DISCUSSION

Stimulation caused lacrimation and in most experiments salivation which indicates that the facial nerve was stimulated. The selectivity of the stimulation is demonstrated by the fact that tissues known to be innervated by the facial nerve responded with clear change in blood flow while control tissues showed no or only minute changes during stimulation. Thus, co-stimulation of other structures interfering with the results seems probable. Nevertheless, if there was a co-stimulation the most suspected structure with regard to the observed results would be the trigeminal nerve. Electrical stimulation of the trigeminal nerve has been shown to cause release of substance P in the eye (Bill et al. 1979) and an increased uveal blood flow and increased blood flow of the upper eyelid and buccal skin (Sjörnschultz, Grøger & Bill 1979). Once transection of the trigeminal nerve was without effect in preventing the vasomotor response to facial nerve stimulation, it may be assumed that no such co-stimulation took place.

Stimulation caused a marked increase in the uveal blood flow, especially in the choroidal blood flow. Since cholinergic as well as ganglionic blockade did not prevent the increase in uveal blood flow the stimulated nerves can hardly be cholinergic nerves with a synapse in the sphenopalatine ganglion; they rather seem to be of an unconventional type. Substance P is unlikely to be the transmitter since this agent causes miosis (Bill et al. 1979) which was not observed in the present experiments. Interestingly nerves containing VIP (Vasoactive intestinal Peptide) have recently been found in several orbital structures in cats. The nerves were in close contact with the small vessels of the choroid, the ciliary body, the lacrimal and tarsal and Harderian glands and VIP immunoreactive cell bodies were found in the pterygopalatine ganglion (Uddman et al. personal communication). VIP causes vasodilation in the choroid and at high doses also salivation (Bill & Näsäon, unpublished results). It seems quite possible then that the unconventional nerves involved in the vasomotor responses reported here are peptidergic using VIP as transmitter.

The mechanism resulting in an increased blood flow in the eyelids, the nictitating membrane and Harderian gland seemed to be different from that in the choroid. Most of the vasodilation in these tissues could be abolished by ganglionic blockade and

by cholinergic blockade. The eyelids of the rabbits contain secretory elements. It is of interest then that in Gautvik's studies (1970a, b, c) the vasodilation obtained in the submandibular gland on chordo-lingual nerve stimulation was due partly to a direct nervous mechanism on the blood vessels and partly to activation of the glandular structures causing release of kinins. Moreover, in recent studies by Uddman et al. (personal communication) release of VIP was observed from the submandibular gland on lingual nerve stimulation. It thus seems that facial nerve stimulation may have direct effect on glands and blood vessels mediated by acetylcholine and VIP and indirect effects mediated by kinins.

Lingual blood flow also markedly increased during stimulation which is in good agreement with the results of Enck & Uvnäs (1957), Eccles & Wallis (1974) and Hellekant (1977). In earlier studies it was not possible to distinguish between the blood flow of the muscle layer and that of the mucous membrane. Thus it was interesting to note in the present experiments that the blood flow in each of the structures increased during stimulation. These nerve fibres may also be peptidergic since ganglionic blockade was ineffective in abolishing the response.

It can be seen from Fig. 1-4 that in some experiments the second flow determination made after a period of 70-115 min with no stimulation indicated flow values on the electrode side that were higher than those on the control side. This result suggests that the period of time without stimulation was not long enough to give a complete return to prestimulation levels. One can speculate that the reason for the poor return in the tongue muscle may be that in tissues with continuous capillaries peptide transmitters with a relatively high molecular weight will tend to have a longlasting effect due to poor elimination via the blood stream.

In the present experiments the intraocular pressure increased moderately during facial nerve stimulation. Gloster (1961) when stimulating the greater superficial petrosal nerve in cats and rabbits also registered a moderate increase in IOP. This effect was resistant to atropine and was thought to be due to vasodilation in the eye. This hypothesis is supported by the present results. The initial rapid increase in IOP in the present study probably was due to vasodilation resulting in an increased intraocular blood volume as well as an increased blood flow. Whether facial nerve stimula-

tion has other effects in the eye remains to be studied

The observation in the present experiments that repeated 3-5 min stimulations were effective is of interest since it indicates that there is no rapid depletion of the transmitter from the nerve terminals. This of course is a prerequisite for the proper function of the peripheral terminals of efferent nerve fibres. Further studies are required however to ascertain whether the nonmuscarinic responses observed in the present study are mediated via afferent or efferent nerve fibres.

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The ultrastructure of the gastric glands and its relation to induced secretory activity of cod *Gadus morhua* (Day)

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Light and electron microscopic studies have been performed on different parts of cod stomach. The studies have been concentrated on the gastric glands of the gastric mucosa. The glands consist of only one cell type. This cell contains an apically located comprehensive tubular system as well as zymogen granules and therefore probably secretes both acid and zymogen. The high degree of development of certain organelles and their internal localization shows a structural organization which appears suitable for an effective secretion. What appears to be intermediate organelle forms are common. When acid secretion is increased by continuous intramuscular infusion of histamine or of carbachol the most marked structural changes are increases in the size of the gland lumen and in the cell membrane facing the lumen. Histamine in particular causes the appearance of numerous long and slender cell processes in an enlarged gland lumen. When the acid secretion increased by histamine had reached plateau metiamide was infused. This histamine H_2 -receptor antagonist caused decrease in acid secretion as well as in the size of the gland cell surface to values characteristic of untreated cod.

Key words: Gastric glands, ultrastructure, acid secretion, cod.

From studies on different organisms it is well known that histamine strongly stimulates gastric acid secretion (Popielski 1920, Keeton et al 1920, Hedman 1937, Gygis et al 1968) and that metiamide reduces or eliminates this effect of histamine (Black et al 1973, Gibson et al 1974, Konrek et al 1974). Recently Holstein (1976) has examined the effect of histamine, metiamide and carbachol on the gastric acid secretion from the common cod *Gadus morhua*. Histamine and carbachol were found to increase the acid output. The effect of both agonists was blocked by metiamide.

The present investigation complements the above mentioned studies on the acid secretion of cod stomach. It comprises light- and electron microscopic studies on different parts of cod stomach. The purposes are: (1) to give the structure and ultrastructure of the cod stomach and (2) to show structural and ultrastructural changes which can be related to a changed acid secretion caused by histamine by a subsequent addition of histamine together with metiamide and by carbachol alone.

The histology of the stomach of cod has been described by several authors (Thesen 1890, Greene 1912, Blake 1930, Rogick 1931, Bishop & Odense 1966). Gastric function in fish has been treated in recent reviews (Kapoor et al 1975, Fluge & Grove 1979). We have found no previous electron microscopic studies on the stomach of cod.

MATERIAL AND METHODS

Cod were kept in aquaria with running sea water for 1-2 weeks before the experiments were performed. The cod were all around 30 cm long. No regard was taken to different sexes as their secretory activities are similar. Untreated fish were used to make comparisons. The experimental fish prepared for collection of gastric secretions as described earlier (Holstein 1979) were treated with continuous intramuscular infusion either of carbachol 5 µg/kg h, or of histamine 15 µg/kg h. For some fish treated with histamine after the secretory rate had attained plateau, metiamide 2.44 mg (10 µmol)/kg h was superimposed on the histamine infusion. Within 1-3 min after stopping the fish samples for fixation were taken from three regions of the internal stomach wall, i.e. the uppermost part and about one third and two thirds down

the stomach length. Our main interest concerned the region one third down the stomach length from the oesophagus.

Histology. Pieces of isolated mucosa were fixed and embedded either by routine methods for light microscopy or as described below for electron microscopy. Staining was done with toluidine blue or with eosin haematoxyline. A modified Zimmermann technique (Marka & Drysdale 1957) was also used.

Electron microscopy. The best fixation was obtained with 3% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.3) made up to a final concentration of 5% with sucrose and adding a trace of CaCl_2 (cf. Bell et al. 1969). Post-fixation was done in 1% OsO_4 in 0.1 M veronal acetate buffer at a pH of 7.4. For some specimens this OsO_4 solution was the only fixative used. Dehydration in increasing concentrations of ethanol was followed by embedding in Epon 812. Sections were cut with an LKB ultratome set at 100 to 500 Å. Contrast was obtained by using uranyl acetate followed by lead citrate. An Hitachi HS-8 electron microscope was used and gave magnifications from $\times 1900$ to $\times 47000$.

RESULTS

Histology. As has been reported by some authors e.g. Blake (1930) the gastric cells of teleosts often do not take or take only lightly the ordinary cytoplasmic stains. In our study the best results were obtained with toluidine blue when used for $\frac{1}{2}$ –1 μm thick sections cut from tissue embedded in Epon 812. Thinner sections used for low power electron microscopy gave complementary information about the microscopic characteristics of the stomach wall.

The layers of the stomach wall are in order mucosa, submucosa, muscle layer and serosa. The gastric mucosa consists of surface epithelium and a connective tissue layer containing the gastric glands.

The surface epithelium is composed of columnar cells which reach their maximum length in the upper and middle part of the stomach. Among the columnar cells are goblet cells which due to their translucent vesicles appear light in the electron microscope and are less densely stained by toluidine blue. Both of these cell types have microvilli, but those of the goblet cells are short and more scattered. The cylindrical epithelium cells when they descend the pits show a gradual transformation to cuboidal neck cells. These latter ones show special staining properties (cf. Leeson 1975a).

Below the epithelium there is a thick connective tissue layer. Within this layer and well separated from the epithelium there is a region containing the gastric glands. The glands converge in groups to-



Fig. 1. Section through stomach wall showing the perimucosa, lumen (l), gastric pits (p) and the glands which occur in a thick connective layer. The glands emerge with common openings into the gastric pits. Epon section, stained with toluidine blue.

wards a common passage which emerges into a pit of the gastric wall (Fig. 1). Among the glands the connective layer is interwoven with blood capillaries and some muscle cells. Within the submucosa no distinct muscle layer is developed.

Electron microscopy. As in most other fish studied (Blake 1930, Rauther 1940, Hale 1965, Li & Tan 1975, Noailiac, Depeyre & Gas 1978) and most other non-mammalian vertebrates (Selverstone 1962, Leeson 1975a, Wight 1975) the gastric glands of the cod contain only one type of cells, supposed to secrete both pepsinogen and hydrochloric acid. This type of cell usually is referred to as oxyntopeptic (Barrington 1942, Smit 1968). In transverse section 5–8 such cells build up each gastric gland (Fig. 2). The cells are pyramidal with flat narrow tops bordering the lumen of the gland. The boundaries between the cells are enlarged and undulated and form spaces between the cells which probably are of importance for the secretory function of the cells (Ito 1961, Kaye et al. 1965).

Within the cells 4 components predominate: vesicles more or less filled with a rather dense homogeneous material, well developed mitochondria,



Fig. 2. Electron micrograph of a transverse section through a gastric gland from untreated cod. The cells are separated by undulating canals. In the gland lumen (l) there are microvilli processes and an electron dense material appearing like the contents of the zymogen granules. The cells are filled up with mitochondria, zymogen granules, and apically arranged smooth tubules. The rough endoplasmic reticulum makes the cell bases more dark. (c) the connective layer blood vessels (b). Fixation: glutaraldehyde overiron.

smooth densely packed tubules and a rough endoplasmic reticulum. The nuclei are located in the basal part of the cells. The vesicles are of about the same size—around $1 \mu\text{m}$ in diameter—and are uniformly scattered over the cell (Fig. 2). Close to the lumen of the gland the vesicles are often seen to release their contents by a process of exocytosis (Fig. 6). It is common to find former vesicular contents in the gland lumen as aggregates mostly enclosed by thin and broken membranes (Fig. 3). Occasionally zymogen granules as such seem to be extruded into the gland lumen (cf. in Fig. 3).

The mitochondria are scattered all over the cell

but are more numerous towards the periphery of the glands. Mostly the mitochondria are densely packed with cristae and generally contain electron dense granules (Figs. 4a–6). In some cases, however, only portions of the mitochondria contain cristae while the rest consists of a relatively dense zymogenlike matrix or is entirely translucent (Fig. 4b–6). Some of these structures often give the impression of being intermediate forms between mitochondria and zymogen granules (cf. Sedar 1961). The high frequency of these structures indicates the existence of transition forms. Cod injected with histamine alone, often show mitochondria with



Fig. 3. Electron micrograph of apical portion of a gland cell from untreated cod. In the lumen a zymogen granule (Z) together with zymogen partly enclosed by a thin membrane. This membrane also fragmentarily borders the lumen. At arrows there are indications of openings of the smooth tubules into the lumen. Glutaraldehyde-osmium.

a reticular conformation of the cristae. Marked mitochondrial changes also occur in glands from animals treated with carbachol: most of the mitochondria show a compartmentalisation and the different regions have their special arrangement of cristae.

Irrespective of the treatment of the cod the smooth tubules predominate in the apical part of the gland cell (Figs. 2 and 5). The smooth tubules often show a very intimate contact with the mitochondria as well as with the zymogen granules. Several surface views give the impression of tubules passing the membranes of these organelles (Figs. 4a, 5, 6). Apically the tubules often show an apparent communication with the gland lumen (Figs. 3, 5). This gives the possibility that acid is added to the gastric juice by an evagination process.

The rough endoplasmic reticulum is well supplied with ribosomes and generally occupies a great portion of the cell basis. Mostly it is in close association with the mitochondria and the zymogen

granules and fills up the space between the ganelles (Fig. 4b). Due to the contrast against the rough endoplasmic reticulum gives the basis a high degree of electron density.

In untreated animals the gland cell surface towards the lumen shows some short processes; the surfaces bordering neighbouring cells are isolated (Fig. 2). The most marked structural change of the gland cells when treated with histamine is an enormous increase of the apical cell surface (Fig. 5). Moreover the gland lumen is increased. A great number of long and thin processes occupy most of the lumen and in some pictures at least about intercellular boundaries show an increased related arrangement. A coincidental reduction of the amount of the smooth tubular system, as reported for several organisms when treated with histamine (Vial & Oregio 1960; Sedar 1961, 1962) is not obvious in glands of histamine-treated cod.

The previous observation that metamide suppresses histamine-induced gastric acid secretion (Holstein 1976) was confirmed in the present investigation. Comparing histamine-treated cod with those injected with both histamine and metamide the latter have a great reduction in surface area towards gland lumen. This surface is of about the same size as that of untreated cod (Fig. 6).

The secreted zymogen occurs as a semi-dense mass in the lumen of the gland. Estimation of the sectioned area of the zymogen in relation to the luminal surface in a number of glands (38) gave an indication of the variation in amount of secreted zymogen. Histamine stimulates acid secretion and increases the size of the gland lumen. In spite of this the zymogen areas did not decrease. This indicates an increase of zymogen secretion also. Using a modified technique in which the fish was supplied with water in the intestine (Holstein 1976; Cederberg (unpublished) by studying the proteolytic activity of the gastric outflow, we found that histamine increased the pepsin activity. A similar increase has been reported for the western newt (Leeson 1975). In fish exposed to a combined treatment with histamine and metamide the increased zymogen secretion obtained with histamine alone often seemed to persist for a period of some hours (Fig. 6).

Cod treated with carbachol also show an increase in gland cell surface though less marked than in those injected with histamine. As reported by Holstein (1976) the increase in acid secretion was less with the concentrations of carbachol used.

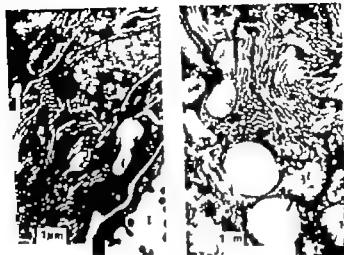


Fig. 4. Portions of gland cells from cod continuously supplied with histamine. (a) At some places (large arrow) one might get the opinion that tubules penetrate into mitochondrion. Often these tubules and organelles show an intimate contact. \downarrow the lamina (\downarrow) the typical thin membranes. (b) Possible intermediate forms between mitochondria and zymogen granules at small arrows. Glutaraldehyde-osmium.

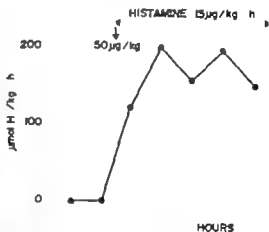


Fig. 5. Apical portions of gland cells of cod treated with histamine. An increase of gland lumen and an enormous increase of cell surface towards lumen are obvious. Apparent close associations between mitochondria and smooth tubules at small arrows. Indications of openings of the tubules into the lumen at large arrows. Graph shows acid secretion against time. Where the curve ends the cod was killed. Glutaraldehyde-osmium.

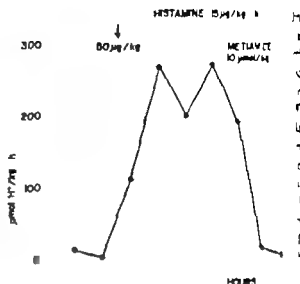
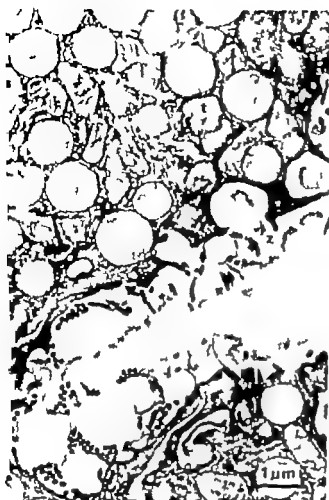


Fig. 6 The lumen region of a gland of cod supplied histamine superposed by metamide. Metamide caused reduction of the number and size of the processes in the lumen. The lumen is filled up with zymogen and membranes. At a zymogen granule empty. The transition form between zymogen granules and mitochondria at *i*. Dense granules occur within several mitochondria and at a translucent region. At arrow smooth tubules seem to enter a granule supplied mitochondria. On gives the variations of acid secretion during the experiment. When the curve ends the cod was killed. Glucose dehydrogenase.

DISCUSSION

The stomach wall of cod is built up of layers in a similar way to that described for mammalian stomach. In cod however there is no distinct muscle layer within the submucosa. Muscle cells occur scattered in the connective layer as has been reported for some other fish (Noallac Depeyre & Gas 1978).

The structure and ultrastructure of the gastric glands of cod show similarities with those of other lower vertebrates. As reported for reptiles (Ferri et al. 1974) for amphibians (Sedar 1967) and for some fish (Western & Jennings 1970; Ling & Tan 1975; Noallac Depeyre & Gas 1978) the cod gastric glands contain one type of cells only. These cells have secretory granules or vesicles as well as a vast tubular system and we suggest that they have the function of secreting both digestive proenzymes and the acid. The vesicles probably contain and release a proenzyme with proteolytic activity. A pepsin activity in gastric juice has been shown in some fish (Norris & Mathies 1953; Norris et al.

1973; Ozguyan et al. 1968; Smit 1967). The same tubular system predominating in the apical portion of the gland cells resembles that of anaplastic "oxyntopeptic" cells and though lacking a cuticula that of mammalian parietal cells. This system seems to open towards the gland lumen and thereby reality to form an immense cell surface. Several authors working with different animals (Kashida et al. 1968; Lillibridge 1968; Sedar 1969; Leese 1977; 1974) have suggested that the tubules for part of the cell membrane. A vast surface of similar kind is common for cells with a high exchange e.g. the chloride cells and some parietal cells of fish (Copeland & Dalton 1959; Pott & Copeland 1963) and it is an attractive hypothesis that the tubules function as a store for acid. We expect that when acid secretion is stimulated by drugs the tubular system would bulge out in the lumen and give rise to the numerous processes characteristic of the stimulated cells. Further studies are planned to test this hypothesis.

The cell surface facing the lumen is covered with

in membrane which generally joins the process and also the zymogen when it is extruded. The membrane appears like the peritrophic membrane cribbed from the intestine of an insect *Trilatoma* (see by Burgos & Gutierrez (1976) Leeson 15) described the occurrence of a membrane glycocalyx or "fuzz" on the microvilli and the apical plasma membrane of the stomach of the Aers newt. He also suggested a release of mitogenic vesicles into the lumen of the gastric gland owing to histamine stimulation. In the cod the membrane enclosing zymogen is generally thin and of the same type as that covering the cell surface though occasionally large zymogen vesicles may near the lumen.

Carlisle et al (1978), working with the isolated mucosa of the haddock *Rana caribbiana* suggested that the theophylline-induced increase in total membrane area is controlled by the cellular level of cyclic AMP. These workers showed that theophylline in the presence of metiamide increased gastric acid secretion and that the luminal surface of the gland cells increased at the same time. Theophylline alone in the state of blockade acid secretion obtained either by anoxia of SCN increased the luminal area of the oxyntic cells. By contrast, the present work, the oxyntic cells revert to resting state in the presence of metiamide, prior to the continuous injection of histamine. Whether these results are contradictory or not depends on the mechanism of action of metiamide. If

metiamide exerts its action by blocking extracellular H_2 -receptors only then it is understandable that the histamine-induced structural changes revert to the resting state (as in the present work). This effect was not obtained with theophylline by Carlisle et al probably because theophylline acts at a site beyond the receptor by inhibiting intracellular phosphodiesterase (Butcher et al 1966) as was supported by Fromm et al (1975) who found that histamine-induced but not theophylline-induced cAMP-induced acid secretion in the isolated rabbit gastric mucosa, was blocked by metiamide. Similar results have been reported by Sjöstrand et al (1978), working with the isolated guinea pig gastric mucosa. By contrast Brown et al (1975), using anoxia from *Rana pipiens* presented evidence that metiamide acts at a step following cAMP. Sjöstrand et al (1978) in the same species found that the acid secretion stimulated by aminophylline or db-cAMP was antagonized by metiamide and concluded that

metiamide exerts its action intracellularly. The results of our study however are best explained by an effect of metiamide on extracellular receptors only.

A high secretory activity of the gland cells is in accordance with the occurrence of special well developed organelles as well as with the close spatial arrangement of some of these organelles. Thus the well developed mitochondria occur close to the apical tubular system indicating the energy demand of the tubules as ion-secreting structures. Well developed mitochondria also occur connected with the rough endoplasmic reticulum whose ribosomes might supply the adjacent vesicles with their contents of pro-enzymes. As to the mitochondria the function of these granules has been discussed (Barnard & Afzelius 1972). Probably the granules form stores of calcium the concentration of which is suggested to be regulatory in acid secretion (Sachs et al 1978). Possibly they represent stores of phospholipids and if so they are probably contributing to the abundance of membranes which aid in the secretion. All the ultrastructural data hence support the occurrence of a high secretory activity of these cells including secretion of acid as well as of proenzymes. Ito & Schofield recently (1978) described how the parietal cells of mouse and cat change their morphology in response to stimulation and emphasized the importance of seeking a better understanding of how the cell structures are involved in the secretion. We believe that by studying different species and getting a broad phylogenetic knowledge it will prove possible to find the structural correlates of gastric secretion.

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Changes in blood volume and extravascular water content in isolated perfused rat lungs during ventilation hypoxia

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Isolated rat lungs were perfused with homologous blood at constant volume inflow. The effect of ventilation hypoxia on pulmonary vascular resistance, preparation weight and reservoir volume (vascular capacitance) were studied. In some experiments also wet/dry weight ratio of the preparation was estimated (extravascular water content). There was no difference in this last parameter between hypoxic and normoxic lungs; thus alveolar hypoxia had no effect on tissue water content as previously described in intact rats. With forward perfusion small and transient changes in either direction were seen in preparation weight and reservoir volume, even though inflow pressure exceeded 5 kPa during alveolar hypoxia. With backward perfusion marked weight increases were seen, and if inflow pressure in this situation was above 3.5 kPa, the weight change was irreversible, thus indicating outward fluid filtration. It is concluded that the vessels responding to alveolar hypoxia are located on the arterial side of the pulmonary vasculature.

Key words: Microcirculation, pulmonary circulation, vascular resistance, lung water content, respiration, lung compliance.

The pulmonary vasoconstrictor response to hypoxia acts to divert blood away from hypoxic to normoxic areas of lung (Hughes 1975). According to Hauge (1969) alveolar hypoxia and not arterial hypoxemia is the main denominator of this vasoconstriction. In concert with these observations, Bjertnæs, Hauge & Torgersen (1978) report that equimolar concentrations of the inhalation anesthetic halothane inhibits the vasoconstrictor response to hypoxia more effectively when administered via the airways than via the pulmonary artery and least via the pulmonary veins. Although distinct anatomical locations can be pointed out, the authors suggest that the hypoxia-sensitive site is located on the arterial side of the pulmonary vasculature and functionally closer to the airways than to the responding blood vessels. Controversies exist, however, about which vascular segment constrict upon hypoxia. Kato & Job (1966) have suggested that the constriction of all pulmonary arteries sufficiently explain the in-

crease in pulmonary vascular resistance during ventilation hypoxia. Others have demonstrated increased venous resistance as well (Fornidal, Ljenden & Snow 1970; Hyman & Kadowitz 1975). Duke (1957) has proposed the increased resistance to be located to the capillaries. Aarseth & Karlsen (1977) claimed that all the consecutive vascular segments are involved. The latter authors observed a marked reduction in blood volume (56%) and in extravascular water content (33%) during ventilation hypoxia in intact rats and postulated that changes in plasma osmolality and/or nervous activity might be responsible.

The purpose of the present work has been twofold: (1) to localize the vascular segments being involved in the pulmonary vasoconstrictor response to ventilation hypoxia and (2) to test whether this mechanism *per se* might be responsible for the reductions in blood volume and extravascular water content observed during ventilation hypoxia in lungs of intact animals.

c 1 The effect of ventilation hypoxia on pulmonary tissue water content in forward perfused isolated lungs

rat lungs, C: control lungs

| | Flow (ml/min) | Baseline P_{PA} (kPa) | ΔP_{PA} (kPa) | ΔP_{PA} (kPa) | Wet/dry weight | P_{TP} (kPa) | ΔP_{TP} (kPa) |
|---|------------------|-------------------------------|--------------------------|--------------------------|-------------------|-------------------|--------------------------|
| T | 12.5 | 1.81 | 2.84 | 3.35 | 5.60 | 1.66 | 0 |
| C | 13.0 | 1.81 | 2.45 | 0 | 6.01 | | |
| T | 13.0 | 1.18 | 2.71 | 3.23 | 5.28 | 1.66 | 0 |
| C | 14.5 | 1.29 | 1.55 | 0 | 6.03 | | |
| T | 1.0 | 1.68 | 1.94 | 2.32 | 5.38 | 1.44 | 0 |
| C | 13.0 | 1.03 | 1.71 | 0 | 5.19 | | |
| T | 12.5 | 2.19 | 4.00 | 4.13 | 5.57 | 1.50 | 0 |
| C | 13.0 | 1.39 | 3.23 | 0 | 5.10 | | |
| T | 14.0 | 1.81 | 2.84 | 3.87 | 4.82 | 0.98 | 0 |
| C | 16.0 | 2.45 | 2.58 | 0 | 5.91 | | |
| T | 15.0 | 2.19 | 2.58 | 2.84 | 4.88 | 1.50 | |
| C | 14.0 | 2.19 | 1.68 | 0 | 4.45 | | |
| T | 17.0 | 2.06 | 1.81 | 2.84 | 6.08 | | |
| C | 15.0 | 1.55 | 2.97 | 0 | 4.67 | | |

Pulmonary arterial pressure responses to hypoxia

Recordings of the pulmonary arterial inflow pressure and the corresponding weight changes of one of the preparations are shown in Fig. 2. Weight changes of about 100 mg were observed during hypoxic pressure responses. When pulmonary arterial pressure increased from 1.86 to 3.06 kPa caused by increased flow or from 1.81 to 3.74 kPa

caused by the injection of 25 μ g bradykinin the weight increased by 300 and 220 mg respectively. Flow reduction with corresponding pressure reduction resulted in decreased weight and increased reservoir volume. In another preparation weight increased 400 mg when inflow pressure was increased from 2.06 to 3.74 kPa by increased flow. Thus the

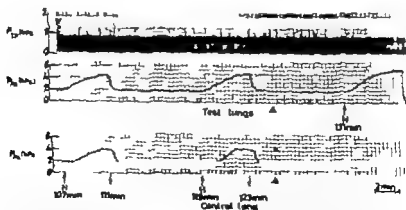


Fig. 1. Upper curves: Transpulmonary pressure (P_{TP}) and pulmonary artery pressure (P_{PA}) of the test lungs. Lower curve: Pulmonary artery pressure of the control lungs. Typical responses to 4 min periods of ventilation hypoxia are elicited in both preparations. ^{51}Cr labelled erythrocytes in 10% albumin was added to the blood reservoir of each preparation before the last hypoxic period of the test lungs (A). The closed circles indicate removal and rapid freezing of both preparations.

METHODS

Wistar rats weighing between 200 and 250 g were used. The operation procedure has been described previously (Bjertnes 1977). The heart-lung preparation was removed from the chest and the pulmonary artery and the left atrium were cannulated. The inflow cannula was connected to a Statam P23Db transducer and an airtap.

The lungs were perfused with heparinized (10 I.U./ml) homologous blood by means of a roller pump; the blood draining back to a thermostated reservoir. Inflow pressure was monitored on a Sanborn Model 2200 six channel recorder and flow was set to give an initial inflow pressure of 1.0–1.5 kPa. Since outflow pressure and flow were kept constant in each experiment, changes in pulmonary vascular resistance were directly reflected as changes in pulmonary inflow pressure. Ventilation which was usually performed with a Starling "Ideal" pump started with 2–3 gentle hyperinflations to a pressure of 2 kPa and the volume was then set to give an initial inflation pressure not exceeding 1 kPa. The frequency was 70 inflations per min. The end-expiratory pressure was 0.15–0.2 kPa. Transpulmonary pressure (P_{TP}) was continuously recorded. The ventilation gas was 5% CO_2 in air for periods of 8 min interposed by repeated 4 min periods of ventilation with a gas mixture containing 2% O_2 , 5% CO_2 and 93% N_2 . During these periods the perfusate was equilibrated with the P_{O_2} of the ventilation gas. A vasoconstrictor response was usually evoked after 2–5 such hypoxic periods.

The lung preparations were suspended underneath a force transducer Model FTA 100-1 (Hewlett Packard) and the weight changes were continuously registered on the Sanborn recorder. In some experiments effluent blood from the lung preparation was drained via a 1 ml syringe mounted on the top of the blood reservoir. Thus reservoir volume changes down to 0.05 ml could be observed continuously with the registration of weight changes. Three series of experiments were performed.

Series I In 7 expts. two lung preparations were forward perfused simultaneously but independently. The preparations were given the same handling and perfusions were started only 5–10 min apart. When the pulmonary pressor response had reached a certain magnitude in both lung pairs, one of them was chosen by random to be the test lung preparation, whereas the other served as control. A suspension of ^{51}Cr labelled rat erythrocytes in ^{25}Mg -serum albumin solution (Institutt for Atomenergi, Kjeller, Norway) was then added to the perfusates, giving an activity of about 80 000 c.p.m. of each isotope per ml. After a mixing period of 6 min, hypoxia was given to the test lungs. At the top of the hypoxic response both preparations were rapidly removed and frozen in liquid nitrogen. From the isotope content of the lung preparations and perfusate samples, the pulmonary blood content was calculated, thus allowing estimation of the wet/dry weight ratio of the lung tissue proper as previously described (Aarseth & Bø 1972). In each experiment, the tissue water content could be compared between the hypoxic and the normoxic preparation.

Series II Forward perfusion of 5 single preparations. Preparation weight and reservoir volume were followed in all experiments during periods with alveolar hypoxia. In

preparations these parameters were followed in 4 periods with increased flow and thereby increased alveolar pressure. In 1 preparation vascular resistance and flow pressure were increased by injection of bradykinin (Sandoz A.G.).

Series III Backward perfusion of 7 single preparations. Preparation weight changes were recorded in all experiments and reservoir volume changes were registered during periods of alveolar hypoxia. In 4 preparations the tissue water content was finally evaluated in the group I expts. Three of these preparations were removed during hypoxia, one during normoxia. For technical reasons it was not possible to include controls in backward perfusion.

For statistical evaluation of the results, Wilcoxon sample test (two-sided) was used.

RESULTS

During the initial part of the perfusion with ventilation hypoxia induced no or only small pressor responses. The number of hypoxic periods varied between 7 and 8 in different preparations before hypoxic pressor responses of more than 1.3 kPa were obtained.

Series I Recordings from one expt. are illustrated in Fig. 1. Increasing pressor responses after repeated periods of ventilation hypoxia were simultaneously evoked in both lung pairs as changes in P_{TP} , indicating that vascular resistance were not influenced (Hauge, Bø & Waaler 1974). In 7 expts. listed in Table 1 median inflow pressure in the test lung preparation was 1.8 kPa (range 1.2–2.19 kPa) with a median flow of 12.5 ml/min (range 12–17 ml/min). The corresponding values for control lung preparations were 1.3 kPa (range 0.9–2.45 kPa) and 14 ml/min (range 13–16 ml/min). During the last hypoxic period the median pressor increase in the test lung preparation was 3.25 kPa (range 2.32–4.13 kPa). No change in P_{TP} was observed in any experiment. The wet/dry weight ratios showed no difference between the test lungs and the control lungs, and thus no effect of ventilation hypoxia on tissue water content was detected.

Series II In 5 preparations with forward perfusion only small and inconstant weight changes were observed during periods with ventilation hypoxia which gave pressor responses between 1.3 and 2.4 kPa. The observed weight changes were paralleled by opposite changes in the reservoir volume. The complete results including flow, baseline pulmonary arterial inflow pressure, pressor responses to hypoxia and the concomitant weight and volume changes are presented in Table 2.

Table 3. The effect of anoxia hypoxia on (dry weight and on changes in preparation weight (ΔW), reservoir blood volume (ΔV); isolated rat lungs during back and perfusion

| | Flow ml/min | Baseline P_{Tr} (kPa) | ΔP_{Tr} (kPa) | ΔW (mg) | ΔV (μ l) | P_{Tr} (kPa) | ΔP_{Tr} (kPa) | % change | % reversibility ΔP_{Tr} | Wet/dry Weight |
|----|-------------|-------------------------|-----------------------|-----------------|-----------------------|----------------|-----------------------|----------|---------------------------------|----------------|
| 13 | | 1.81 | 0.76 | 125 | | 1.37 | 0.04 | 3 | 100 | |
| | | 1.55 | 0.45 | 0 | | 1.35 | 0.04 | 3 | 100 | |
| | | 1.68 | 1.55 | 250 | | 1.47 | 0.12 | 8 | 100 | |
| | | 1.55 | 1.81 | 0 | | 1.49 | 0.1 | 8 | 100 | |
| | | 1.55 | 3.48 | | | 1.51 | 0.10 | 7 | 100 | |
| | | 1.55 | 3.48 | | | 1.70 | 0.06 | 4 | No | 8.87 |
| | 97 | 3.48 | | | | | | | | |
| 15 | | 1.68 | 0.90 | 875 | | 1.32 | 0.08 | 9 | 75 | |
| | 3 | 0.90 | 1.55 | 925 | - | 1.50 | 0.16 | 10 | 100 | |
| | 3 | 1.03 | 1.55 | 1200 | | 1.57 | 0.18 | 11 | 100 | |
| | 3 | 1.03 | 1.68 | 1300 | | 1.65 | 0.1 | 7 | No | 5.62 |
| | | | | | | | | | | |
| 16 | | 1.55 | 1.16 | 1000 | | 1.72 | 0.18 | 10 | 50 | |
| | | 1.29 | 0.39 | 200 | | 1.80 | 0.08 | 4 | 100 | |
| | | 1.79 | 0.52 | 250 | | 1.80 | 0.08 | 4 | 100 | |
| | | 1.29 | 1.19 | 1500 | - | 1.80 | 0.1 | 7 | 100 | |
| | 14 | 3.35 | | 3700 | | 1.80 | 0.12 | 7 | No | 9.32 |
| 7 | | 1.94 | 0 | 0 | | | | | - | |
| | | 1.55 | 1.55 | 0 | | | | | | |
| | | 1.55 | 0.77 | 250 | | | | | | |
| | | 1.47 | 1.55 | 500 | | | | | | |
| | | 1.42 | 0.06 | 900 | | 1.29 | 0.20 | 15 | 80 | |
| | | 1.42 | 2.45 | 600 | | 1.37 | 0.20 | 14 | N | 6.19 |
| | | | | | | | | | | |
| 10 | | 1.16 | 1.16 | 800 | 650 | | | | | |
| | | 1.29 | 1.16 | 750 | 600 | | | | | |
| | | 1.29 | 1.42 | 850 | 700 | | | | | |
| | | 1.29 | 0.06 | 1050 | 1000 | | | | - | |
| | | 1.42 | 0.06 | 1000 | 900 | - | - | | - | |
| 10 | | 1.29 | 1.42 | 700 | 700 | 1.41 | 0.06 | 4 | 100 | |
| | | 1.42 | 2.19 | 1175 | 1050 | 1.14 | 0.20 | 17 | 40 | |
| 13 | | 1.29 | 1.55 | 572 | 400 | 0.94 | 0.18 | 19 | 50 | |
| | | 1.16 | 1.32 | 803 | 700 | 1.29 | 0.16 | 1 | 100 | |
| | | 1.29 | 2.97 | 1034 | 900 | 1.97 | 0.14 | 10 | 100 | |
| | | 1.29 | 3.23 | 1100 | 1000 | 1.47 | 0.10 | 7 | 100 | |
| | | 1.29 | 3.74 | 1760 | 1550* | 1.57 | 0.39 | 25 | No | |

Isolated flooding

Isolated flooding hypoxia, 4 min in all other hypoxic periods

*and ΔP_{Tr} are increases in pulmonary venous outflow pressure and transpulmonary pressure respectively

maximum reversible increments in P_{Tr} (ΔP_{Tr}) during hypoxia (Table 3) was 0.12 kPa (range 0.06–0.18 kPa) starting from a median P_{Tr} of 1.49 kPa (range 1.29–1.80 kPa).

DISCUSSION

In pulmonary vasoconstriction in response to hypoxia per se cause a reduction in blood volume and tissue water content of the lungs? We have tested this hypothesis by the use of two independent experimental procedures. The double isotope technique (Aarseth & Bo 1977) showed no difference in wet/dry weight ratio during forward perfu-

sion between the test lungs and the control lungs (series I). Since the lungs had to be removed from the experimental upset in order to be frozen in liquid nitrogen, redistribution of minute amounts of blood from the lungs into the outflow tubings might have taken place. A second series of expts. therefore was carried out (series II) in which changes in preparation weight and reservoir volume could be continuously registered. In these preparations the weight changes either were small absent or biphasic consisting of a weight reduction followed by an increase. The weight changes were accompanied by volume changes in the opposite direction but of a corresponding magnitude, which proved

Table 2 Changes in preparation weight (ΔW) and reservoir blood volume (ΔV) during ventilation hypoxia in forward perfused isolated rat lungs

| Expt. no | Flow (ml/min) | Baseline P (kPa) | ΔP_p (kPa) | ΔW (mg) | ΔV (μ l) |
|----------|---------------|------------------|--------------------|-----------------|-----------------------|
| 8 | 15 | 19 | 2.19 | -100 | +100 |
| 9 | 10 | 2.32 | 1.29 | 0 | 0 |
| 10 | 1 | 2.58 | 1.81 | 0 | 0 |
| 11 | 15 | 2.06 | 2.45 | -50/ +100 | +50/ -100 |
| 12 | 11 | 1.81 | 2.45 | +30 | -30 |

Pulmonary arterial pressure

weight changes seen during hypoxia are not only passively due to pressure changes per se

Series III 7 preparations were retrogradely perfused through the left atrial cannula. Tracings of the transpulmonary pressure (P_{TP}), the weight changes (ΔW) and the pulmonary venous inflow pressure (P_{PV}) of one representative expt (No. 19) are shown in Fig. 3. The hypoxic pressor responses during backward perfusion were comparable with those seen during forward perfusion. However during backward perfusion concomitant increases in transpulmonary pressure and in the preparation weight were seen during the hypoxic periods. P_{TP} changes were completely reversible upon cessation of ventilation hypoxia. The initial weight was not regained however and the remaining weight gain was larger with increasing pressor responses. When a certain response magnitude was exceeded pul-

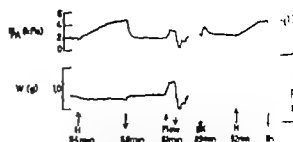


Fig. 2 Simultaneous recordings of pulmonary pressure and weight changes in forward perfused rat lungs. Effects on lung weight of pulmonary pressure increases in response to hypoxia, to flow and to injection of bradykinin are demonstrated.

monary edema developed and neither of changes returned to baseline (last sequence Fig. 3). The same weight response was seen if flow thereby transmural pressure was reduced (expt 14).

The complete results from this series of 6 are listed in Table 3. The wet/dry weight ratio examined in 4 of the preparations. In 2 preparations (no. 13 and 15) with pressor responses of 3.4 and 3.35 kPa respectively the wet/dry weight were markedly increased. In 2 other preparations (no. 14 and 16) modest pressor responses were seen and in these preparations normal tissue content was found. In the 3 expts (no. 17, 18, 19) in which volume changes were measured weight increases during pressor responses during hypoxia were paralleled by corresponding increases in the reservoir volume. The median value

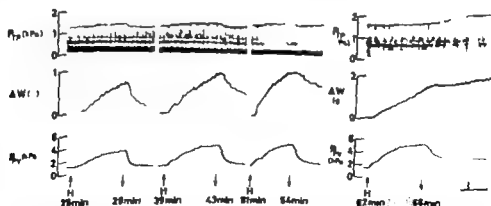


Fig. 3 Simultaneous recordings of transpulmonary pressure (P_{TP}), lung weight change (ΔW) and pulmonary venous pressure (P_{PV}) during backward perfusion of an isolated rat lung preparation (exp. no. 19). The arrows indicate start and termination of ventilation hypoxia (F_{IO_2} 0.02). Time is given in min from start of perfusion. The reversibility of weight changes decreased with increasing pressure responses. The changes in P_{TP} became irreversible (first when alveolar flooding occurred (last sequence)).

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that the fluid volume of the lungs really changed. The rapid off-response upon reversion of the pressor response to hypoxia shown in Fig. 2 also points to an intravascular volume change. The change in tissue water content observed in series I and the changes in fluid volume observed in series II were randomly distributed and thus no effect of hypoxia on lung blood volume and tissue water content could be demonstrated in isolated rat lungs. These findings contrast the marked reductions reported from experiments in intact animals by Aarseth & Karlsen (1977). The discrepancy between the observations in the two studies support the hypothesis that changes in plasma osmolality to a great extent might have been responsible for the lung tissue dehydration *in situ* although nervous mechanisms have not been ruled out.

A problem of considerable physiological interest has been to localize the effector site of the pulmonary vasoconstrictor response to hypoxia. Intuitive ly the most logical way of producing high altitude pulmonary edema would be to increase the pulmonary capillary pressure. Actually there has been presented evidence for increased pulmonary venous resistance during hypoxia in the dog (Rivera Estrada et al 1958), Furnival, Linden & Snow (1970) and in the lamb (Hyman & Kadowitz 1975). Severinghaus (1971) however suggests that arterial leakage takes place during hypoxia whereas Hultgren & Grover (1968) have experience for uneven distribution of vasoconstriction which might lead to relative overperfusion and edema development in some regions of the lungs and underperfusion in others.

The blood content of intact rat lungs has been calculated to be about 1 ml (Aarseth & Karlsen 1977). When the inflow pressure in backward perfused rat lung preparations (series III) increased to a level below about 3.5 kPa during hypoxia, lung weights increased reversibly with 100–150% of this amount, indicating vascular distension upstream to an increased resistance. Simultaneous with the rise in inflow pressure, compliance decreased reversibly by maximum 17% (expt no. 19). Such a reversible compliance reduction has also been shown to be associated with pulmonary blood volume increments during left atrial pressure elevations in the cat (Hauge, Bo & Aarseth 1977) and in the isolated rabbit lung preparation (Hauge, Bo & Waaler 1975). When however inflow pressure during backward perfusion rised to a level above about 3.5 kPa in

response to hypoxia, frank pulmonary edema developed (Expt no. 13, 15 and 19) and the reductions in compliance became irreversible (sequence Fig. 3). In contrast, no significant filtration occurred during forward perfusion when inflow pressure exceeded 5 kPa during ventilation hypoxia (Table 1).

In conclusion, the considerable weight gain during pressor responses to hypoxia in backward perfused lungs, including outward filtration, if the plasma colloid osmotic pressure exceeded the capillary transmural pressure, and the absence of these changes during forward perfusion strongly suggests that vasoconstriction in response to hypoxia is located to precapillary vessels. This well with the hypothesis that the receptor area for hypoxia-induced vasoconstriction is situated on arterial side of the pulmonary vasculature (Bjertnæs, Hauge & Torgersen 1978).

Further experiments, however, must be carried out to investigate whether enhanced plasma osmolality or increased sympathetic nervous activity is responsible for the reductions in blood volume and tissue water content observed during hypoxia in intact rat lungs.

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Old post ejaculatory inhibitory effect of seminal plasma on sperm nuclear chromatin decondensation ability in man

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KVIST U. Rapid post-ejaculatory inhibitory effect of seminal plasma on sperm nuclear chromatin decondensation ability in man. *Acta Physiol Scand* 1980, 109: 69-72. Received 30 Aug. 1979. ISSN 0001-6777. Reproductive Physiology Unit, Department of Physiology, Karolinska Institutet, Stockholm, S. eden

The stability of the nuclear chromatin in human spermatozoa soon after ejaculation was studied by exposing the cells to sodium dodecyl sulphate (SDS) one to 20 min after ejaculation. Semen samples were obtained both from men with apparently normal, and from men with impaired prostatic secretion (=subnormal seminal plasma [Zn]). The sperm nuclear resistance to decondensation in SDS increased in both groups during the first 15 min after ejaculation but was significantly lower in the semen samples with subnormal [Zn]. This fast post-ejaculatory increment in sperm SDS resistance was significantly reduced by 5-fold saline dilution of the semen at the time of ejaculation. It is discussed if the observed stabilisation with time was illusory and that prostatic component instead contributed to intrinsic nuclear chromatin decondensation (NCD) process initiated by SDS disruption of spermatozoal membranes.

Key words. Spermatozoa, human, chromatin stability, seminal plasma, prostate, nuclear chromatin decondensation, zinc

maturation spermatozoal chromatin is apparently stabilized by the formation of disulphide bridges (Mencher 1878 cf Bedford 1975). Support for this concept is the observation by Bedford and co-workers that spermatozoal chromatin stability increases during the epididymal passage also that this increase is concomitant with a release of free thiol (SH) groups in the nucleus (Ivin & Bedford 1970, 1971; Bedford, Bent & Calvin 1973; Calvin, Y. & Bedford 1973).

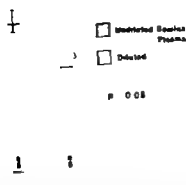
The efficacy of nuclear swelling during a combined treatment with dithiothreitol (DTT) and sodium dodecyl sulphate (SDS) is one way to assess sperm chromatin stability (Calvin & Bedford 1971). According to these authors such a treatment causes decondensation of spermatozoal nuclear chromatin since DTT disrupts S-S bonds (Cleland 1964). If SDS disrupts not S-S stabilized spermatozoal structures (e.g. plasma membranes) Bedford et al (1973) observed that a rather high percentage of spermatozoa from some men also swelled in SDS medium when exposed for 60 min. The latter observa-

tion was recently confirmed (Kvist & Eliasson 1980). It was then also noted that SDS sensitive spermatozoa occurred more frequently in semen samples with a subnormal zinc/fructose ratio and that brief (5 min) exposure of such spermatozoa to exogenous seminal plasma with normal level of zinc and other prostatic components increased their resistance to SDS. Hence these observations indicated that some seminal plasma component of prostatic origin either stabilized spermatozoal chromatin or preserved its stability. It was therefore of interest to study if such seminal plasma components influenced spermatozoal chromatin stability already within 20 min after ejaculation.

MATERIAL AND METHODS

Semen specimens were obtained from 11 volunteers. All specimens were collected by masturbation after 3-5 days of continence. 8 donors are known to have normal composition of the seminal plasma as judged by the biochemical markers for prostatic secretion (Zn > 1.2 mM, Mg > 2.9 mM and acid phosphatase activity > 6.9 mKat/l), and for

before SDS exposure 20



P < 0.05

Reduction of post-ejaculatory human sperm resistance to SDS (sodium dodecyl sulphate) after saline (BSS) dilution of the semen at the time of isolation when the spermatozoa subsequently were mixed to SDS ($P < 0.05$).

groups had higher resistance to SDS when served in seminal plasma from one vs 15 min after ejaculation (P -values for both groups < 0.01). However the spermatozoa ejaculated in seminal plasma with subnormal prostatic contribution (group I) were less stable than spermatozoa in group II both at one ($P < 0.05$) and at 15 min (< 0.01) after ejaculation. In fact the latter group revealed almost maximal spermatozoal chromatin stability (mean 97%) after 15 min in seminal plasma, whereas the corresponding figure for group III was 66%.

Effect of post-ejaculatory milieu

The effect of the surrounding milieu at the time of isolation was investigated in a series of experiments where three donors delivered 2 semen samples each and a fourth donor delivered 4 semen samples (i.e. $n = 10$). Half of the samples from each donor served as controls and were allowed to liquefy for 30 min after ejaculation in their own seminal plasma. An aliquot of each of these samples was then subjected to SDS treatment. Five other samples were ejaculated in boxes containing four volumes of 37°C BSS and at once mixed with the BSS. After 20 min aliquot of these samples were also subjected to SDS treatment. The spermatozoa exposed to the BSS diluted seminal plasma revealed significantly lower resistance to SDS than spermatozoa in the undiluted seminal plasma ($P < 0.05$) (Fig. 1).

DISCUSSION

The effect of anionic detergents like SDS on spermatozoal structures has to be considered before discussing the value of the SDS test as an index of the prevailing sperm nuclear chromatin stability. Apparently SDS per se does not to any appreciable extent cause the disruption of S-S bonds reported necessary to induce nuclear chromatin decondensation. However SDS in a few minutes makes possible the complete swelling of spermatozoa which at the onset of SDS exposure contains nuclear chromatin not fully stabilized by S-S bonds (Bedford, Calvin & Cooper 1973). Nonetheless, in a previous study it was observed that a high proportion of ejaculated spermatozoa from some men underwent progressive swelling during 60 min of SDS exposure (Kvist & Eliasson 1980). It indicates that a process involving gradual disruption of essential chemical bonds in the chromatin (e.g. S-S bonds) under certain circumstances may take place in the spermatozoa, and that this process perhaps is initiated by the SDS-induced derangement of spermatozoal membrane structures. In the study by Kvist & Eliasson (1980) it was also found that the highest proportion of spermatozoa swelling during 60 min in SDS was present in semen with subnormal zinc/fructose ratio and that brief exposure of such unstable spermatozoa to seminal plasma with normal prostatic secretory contribution counteracted swelling in SDS.

The results of the present study provide further support for the idea that a factor in seminal plasma of prostatic origin has the ability to protect ejaculated spermatozoa against swelling in SDS and suggest that this protection is rapidly established. Thus, a considerable increase of the spermatozoal chromatin resistance against swelling in SDS took place already during the first 15 min after ejaculation (Fig. 1A) and semen from men with apparently normal prostatic secretion contained a much larger proportion of SDS resistant spermatozoa than did semen from men with impaired prostatic function at one as well as at 15 min after ejaculation (group II vs group I in Fig. 1B). Further evidence that normal concentrations of some component in the seminal plasma are needed to induce high swelling resistance in SDS is provided by the present observation that immediate post-ejaculatory saline dilution of the semen significantly increased the percentage of spermatozoa swelling during SDS exposure.

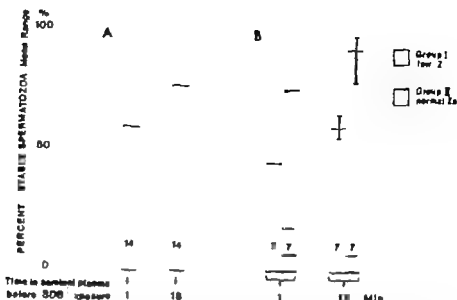


Fig. 1 Increase in human sperm nuclear resistance to decondensation in sodium dodecyl sulphate (SDS) during the first 15 min after ejaculation. A = 14 samples from 7 donors both from men with apparently normal prostatic secretion and from men with impaired prostatic function ($P < 0.001$). B = A subdivision of the material into a low zinc group (I) (zinc conc. 0.4–0.7 mM (I)) and a normal zinc group (>1.2 mM (II)). Significances for differences: Group I 1 min vs. 15 min $P < 0.01$; Group II 1 min vs. 15 min $P < 0.01$; Group I vs. group II 1 min $P < 0.05$; Group I vs. group II 15 min $P < 0.01$.

seminal vesicular secretion (fructose >6.7 mM) (cf. Eliasson 1975). Three donors were known to have prostatic hyposecretion (i.e. subnormal concentrations of prostatic components). Zinc and magnesium in seminal plasma were measured with an atomic absorption spectrophotometer (cf. Eliasson & Lindholmer 1971, 1972). Acid phosphatase activity was determined according to Sigma Technical Bulletin No. 104 (1975) and fructose concentration with a colorimetric technique (Harvonen & Malm 1956) as described by Eliasson (1965).

To reduce the possible influence of seminal plasma components on the nuclear chromatin stability during the first 20 min after ejaculation (i.e. during the gel-phase before complete liquefaction), semen was in some experiments diluted at the time of ejaculation. Dilution was obtained by collecting the ejaculate in a plastic box containing 37°C buffered salt solution (BSS) to the amount of four times the expected semen volume. The composition of BSS was (mM): NaCl 134, KCl 4, HCl 4, TRIS 37, pH 8.0.

Spermatozoal nuclear chromatin stability was assessed as the degree of nuclear swelling after the spermatozoa had been exposed for 60 min to 1% of sodium dodecyl sulphate (SDS) in 0.05 borate buffer, pH 9.0.

One volume of native or diluted semen was mixed with nine volumes of the SDS solution and the reaction was stopped by adding an equal amount of 2.5% glutaraldehyde in 0.05 M borate buffer, pH 9.0. Nuclear swelling was assessed with phase contrast microscopy (500 \times) and the spermatozoa were classified as stable (=not swollen) and unstable (moderately and grossly swollen) (Kvist & Eliasson 1980).

For descriptive statistics mean and range are given. Percentage stable (=non-swelling) spermatozoa and seminal plasma zinc concentrations. Analytical statistics performed by using the Randomization Test for Multiple Paired and the Mann-Whitney U Test for independent groups (cf. Siegel 1956).

RESULTS

Effect of postejaculatory time

Fourteen semen specimens from seven donors were used to find out if spermatozoal resistance to SDS increased already during the first 15 min after ejaculation. Three of the donors (7 specimens) had seminal plasma zinc values between 0.4–0.7 mM; seminal plasma zinc values of the other seven specimens were 1.4–3.5 mM. Aliquots of each semen specimen were exposed to SDS at 1 and 15 min after ejaculation. As shown in Fig. 1A spermatozoa which had remained in their seminal plasma for 15 min had considerably higher resistance to SDS than those exposed to SDS at 1 min after ejaculation ($P < 0.001$). At the same time the range of percentage stable spermatozoa was narrower.

A subdivision of the material into two groups with reference to subnormal (I) and normal (II) seminal plasma zinc values (Fig. 1B) revealed that

Reversible inhibition of nuclear chromatin decondensation (NCD) ability of human spermatozoa induced by prostatic fluid

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In semen from donors with adequate secretory function of the prostate, spermatozoa in the first ("prostatic") portion of the ejaculate were more resistant to nuclear swelling in sodium dodecyl sulphate (SDS) than spermatozoa from the second ("vesicular") portion. No such difference was revealed by donor with severely impaired prostatic function. This demonstrates that some sperm nuclear chromatin stabilizing factor(s) is present in normal prostatic fluid. The chromatin stabilizing factor(s) could largely be removed by washing the spermatozoa in saline containing albumin. Spermatozoa sensitized to SDS in this manner regained their SDS resistance upon exposure to normal (zinc-rich) prostatic fluid. Such exposure also induced a high degree of resistance in actively SDS sensitive spermatozoa. The possibility is discussed that zinc of prostatic or other origin reversibly inhibits a nuclear chromatin decondensation ability (NCD-ability). It is suggested that such a mechanism may be of essential importance for male genetic transfer.

Key words: Spermatozoa, human, chromatin stability, seminal plasma, prostate, nuclear chromatin decondensation, zinc.

Human semen sometimes contains a relatively high percentage of spermatozoa reacting with nuclear chromatin decondensation (i.e. swelling) when exposed for 60 min to the anionic detergent sodium dodecyl sulphate (SDS) (Bedford, Bent & Calvisi 1971; Kvist & Eliasson 1980). Kvist & Eliasson (1980) also found that semen samples with an abnormally or relatively low content of prostatic markers (subnormal zinc/fructose ratio) contained significantly more spermatozoa swelling in SDS than semen samples from men with apparently normal secretory function. Furthermore, spermatozoa from men with an impaired prostatic function became markedly more resistant to SDS when they were briefly exposed to seminal plasma of normal composition. Further evidence that a seminal plasma component of prostatic origin inhibits nuclear chromatin decondensation and thus contributes to preservation of post-ejaculatory chromatin stability has subsequently been presented (Kvist 1980) and it was suggested that prostatic compo-

nent (possibly zinc) may normally counteract an intrinsic nuclear chromatin decondensation (NCD) process initiated by a SDS derangement of spermatozoal membranes.

The split ejaculate technique (Lundquist 1949; Eliasson 1965) provides a tool to separate semen fractions with predominantly prostatic contribution to the seminal plasma from fractions in which the plasma mainly consists of fluid from the seminal vesicles. Therefore with the split ejaculate technique it has now been possible to obtain further support for the concepts that a factor of prostatic origin contributes to preserve the integrity of human spermatozoal chromatin by reversibly inhibiting of NCD-ability.

MATERIAL AND METHODS

Semen specimens were obtained from men referred to the laboratory for barren union, and from volunteers (mainly medical students). All semen samples were collected by masturbation after 3-5 days of continence. Split ejaculates

(Fig. 7). The nature of the active components in the seminal plasma remains to be elucidated. However, the observed co-existence of a reduced spermatozoal resistance to SDS and a subnormal seminal plasma zinc concentration and the preliminary observations that zinc could increase sperm SDS-resistance (Eliasson & Kvist 1976, Kvist & Eliasson 1978) makes zinc worth further consideration.

Appreciable amounts of zinc is generally incorporated into mammalian spermatozoa before they reach the prostate but there are also indications that zinc in human and dog spermatozoa can be derived from the prostate (Janick, Leitz & Whitmore 1971, Saito, Leitz & Bush 1967). Thus zinc may contribute to establish a resistance to SDS in spermatozoa prior to ejaculation.

The possibility that zinc or some other component in seminal plasma acts by inhibiting an intrinsic sperm nuclear chromatin decondensation (sperm NCD) process triggered by a SDS induced derangement of spermatozoal membranes is suggested and warrants further investigations.

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Key words: Spermatozoa, human chromatin stability, seminal plasma, prostate, nuclear chromatin decondensation, zinc.

Human semen sometimes contains a relatively high percentage of spermatozoa reacting with nuclear chromatin decondensation (i.e. swelling) when exposed for 60 min to the anionic detergent sodium dodecyl sulphate (SDS) (Bedford, Bent & Calvin 1973; Kvist & Eliasson 1980). Kvist & Eliasson (1980) also found that semen samples with an abnormally or relatively low content of prostatic markers (i.e. abnormal zinc/fructose ratio) contained significantly more spermatozoa swelling in SDS than semen samples from men with apparently normal prostate function. Furthermore, spermatozoa from men with an impaired prostatic function became markedly more resistant to SDS when they were briefly exposed to seminal plasma of normal composition. Further evidence that a seminal plasma component of prostatic origin inhibits nuclear chromatin decondensation and thus contributes to preservation of post-ejaculatory chromatin stability has subsequently been presented (Kvist 1981) and it was suggested that a prostatic compo-

nent (possibly zinc) may normally counteract an intrinsic nuclear chromatin decondensation (NCD) process initiated by a SDS derangement of spermatozoal membranes.

The split ejaculate technique (Lundquist 1949; Eliasson 1965) provides a tool to separate semen fractions with predominantly prostatic contribution to the seminal plasma from fractions in which the plasma mainly consists of fluid from the seminal vesicles. Therefore with the split ejaculate technique it has now been possible to obtain further support for the concepts that a factor of prostatic origin contributes to preserve the integrity of human spermatozoal chromatin by reversibly inhibiting of NCD-ability.

MATERIAL AND METHODS

Semen specimens were obtained from men referred to the laboratory for fertility reason, and from volunteers (mainly medical students). All semen samples were collected by masturbation after 3-5 days of continence. Split ejaculates

Table I Percentage of SDS resistant spermatozoa and zinc concentration (mM) in prostatic portion and vesicular portion (II) of split-ejaculates from 5 donors with apparently normal prostatic function (Group A) and one donor (5 samples) with impaired prostatic function (Group B). Mean and range

| Fraction | Percentage stable spermatozoa | | | Portion zinc concentration | | |
|----------------|-------------------------------|---------|---------|----------------------------|-----------|-----------|
| | I | II | A | I | II | A |
| <i>Group A</i> | | | | | | |
| Mean | 77 | 60 | 18 | 0.9 | 0.7 | 0.2 |
| Range | (61-99) | (37-83) | (17-74) | (1.5-5.7) | (0.1-1.6) | (1.1-4.1) |
| <i>Group B</i> | | | | | | |
| Mean | 58 | 61 | 3 | 0.5 | 0.4 | 0.1 |
| Range | (52-63) | (56-66) | (-5-7) | (0.4-0.7) | (0.3-0.6) | (0.0-0.3) |

$P < 0.05$

Not significant.

were generally collected in 8 fractions which afterwards were pooled into two portions (Lundquist 1949). Portion I consisted of the 3 first fractions (seminal plasma with mainly prostatic contribution) and remaining fractions (with high seminal vesicular contribution) were pooled as Portion II. Occasionally experienced donors were instructed to collect the ejaculate directly in the two portions mentioned. This proceeding was for example used in experiments involving an immediate post-ejaculatory five-fold saline dilution of the semen according to the technique previously described (Kvist 1960).

Spermatozoa were studied either in aliquots of the semen portions or after having been concentrated by mild centrifugation (400 g for 15 min) followed by removal of the supernatant. Washed spermatozoa were prepared by washing the centrifugation pellets twice in either a magnesium free buffered salt solution (BSS) or in BSS containing 4% human albumin (BSSA). The composition of BSS was (mM): NaCl 123, KCl 5, HCl 4, TRIS 37 (pH 8.0). Seminal plasma from Portion I ("prostatic fluid") and from Portion II ("vesicular gland fluid") were obtained by centrifugation (2400 g for 70 min) of the two portions and collection of the supernatants.

Nuclear chromatin stability was assessed from the degree of sperm head swelling after 60 min exposure to 1% sodium dodecyl sulphate (SDS) in 0.05 M sodium borate buffer (pH 9.0). One volume of semen or sperm suspension was mixed with 9 volumes of the SDS solution and the reaction was stopped by adding the equal amount of 0.5% glutaraldehyde in 0.05 sodium borate buffer (pH 9.0). The degree of nuclear swelling was then semi-quantitatively evaluated by phase contrast microscopy (500 \times) and scored as previously described (Kvist & Eliasson 1960).

The secretory contribution of the prostate was assessed by measuring the seminal plasma zinc and magnesium concentrations with an atomic absorption spectrophotometer (cf. Eliasson & Lindholmer 1971, 1972) and its acid phosphatase activity (cf. Sigma Technical Bulletin No. 104). An index of the vesicular gland secretory contribution was obtained by colorimetric determination of the

seminal plasma fructose concentration (Kjorset & Mahn 1955) as described by Eliasson (1965).

For descriptive statistics mean and range is given. Determined percentage of SDS resistant spermatozoa for the chemical variables. Analytical statistics was performed with the Randomization Test for Matched P (cf. Siegel 1956).

RESULTS

A Albumin induction of sperm VCD-ability

Spermatozoa from 6 semen samples (obtained from different donors) were subjected to washing 3 times after ejaculation. One half of each spermatozoa population was washed in BSS and the other half were washed in BSSA. The spermatozoa were then subjected to SDS exposure for 60 min. Those samples washed in BSS contained on the average 9% stable spermatozoa while those washed in BSSA had a mean percentage stable spermatozoa of 14 (mean difference 8, range 0-18, $P < 0.05$). However in a corresponding series of experiments (5 semen samples from different donors) the spermatozoa were washed in BSS or BSSA already 15 min after ejaculation and the differences were considered greater. The mean percentage stable spermatozoa of aliquots washed in BSS was 5% and of those washed in BSSA 35 (mean difference 21, range 1-39, $P < 0.05$).

B Difference in sperm VCD-ability between split-ejaculate portions

Five donors with previously observed normal composition of the seminal plasma and one donor with

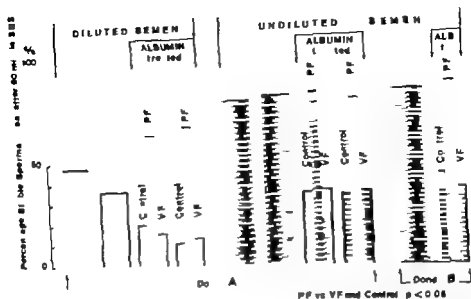


Fig. 1. Restoration of nuclear chromatin stability of experimentally destabilized spermatozoa by the exposure to dogenous prostatic fluid (PF). Endogenous vesicular fluid (VF) lacks this effect. Nuclear destabilization was induced by immediate post-ejaculatory saline dilution of the semen and/or washing in albumin containing saline 20 min after ejaculation.

ALB: albumin washed and albumin exposed spermatozoa

SDS: Sodium dodecyl sulphate

Both donors had apparently normal prostatic function. For further details see text.

diagnosed impairment of the prostatic function (inverted split-ejaculates as portions I and II). The 5 normal donors provided one sample each, whereas the donor with the impaired prostatic function delivered 5 samples. Within 30 min after ejaculation the spermatozoa in each portion were subjected to SDS exposure for 60 min.

As shown in Table 1 the samples from the normal donors contained a significantly higher percentage of SDS resistant spermatozoa in the zinc-rich portion I ("prostatic dominated portion") than in the zinc-poor portion II ("vesicular fluid dominated portion"). In contrast, the semen samples from the donor with impaired prostatic function did not reveal any difference in the percentage SDS resistant spermatozoa between portions I and II, where the zinc concentration in portion I was almost as low as in portion II (Table 1).

Effect of prostatic fluid on sperm NCD-ability

Experimentally induced SDS sensitivity. The effect of prostatic fluid vs. vesicular fluid on the chromatin decondensation ability was studied on

experimentally SDS-sensitized spermatozoa. Increased SDS-sensitivity was obtained by an immediate post-ejaculatory 5-fold saline dilution of the ejaculate and/or by washing the spermatozoa twice with BSSA. The washing procedure was started 20 min after ejaculation. Semen samples were obtained from donors A and B with known apparently normal prostatic function. Donor A delivered 8 ejaculates, 4 of which were collected as whole semen, and the remaining four as split ejaculates in 2 portions (I and II) (see Methods). Two of the split ejaculates, and two of the whole ejaculates were immediately subjected to BSSA dilution. Prostatic and vesicular fluids (diluted or undiluted) were obtained by centrifugation of portions I and II from all split ejaculates 20 min after ejaculation, and the spermatozoa of portions I were then washed with BSSA. Aliquots of these albumin treated spermatozoa were incubated (60 min) in either BSSA "prostatic fluid" or vesicular fluid (Control PF and VF in Fig. 1), and then exposed to SDS. Spermatozoa from the two diluted and the two undiluted whole ejaculates were exposed to SDS 20 min after ejaculation.

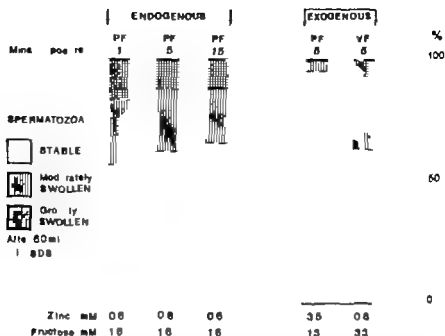


Fig 2 Left Low SDS (sodium dodecyl sulphate) resistance of spermatozoa from a man with impaired prostatic function. The spermatozoa were preserved for 1 to 15 min after ejaculation in his own zinc-poor "prostatic fluid" (PF). Right: Markedly increased SDS resistance induced in an aliquot of the same sperm population after 5 min exposure to exogenous zinc-rich PF from a donor with apparently normal prostatic function. Corresponding exposure to "vesicular fluid" (VF) from this donor had no obvious effect on the spermatozoal SDS resistance.

Donor B delivered one whole and one split ejaculate; none of them diluted. These ejaculates were treated in the same manner as the undiluted ejaculates of donor A, with the exception that the incubation period (in Control PF and VF) was reduced to 5 min.

The sperm resistance to swelling in SDS under various experimental conditions is shown in Fig. 1. The immediate post-ejaculatory saline dilution of semen from donor A caused a reduction of the percentage SDS resistant spermatozoa. Albumin treatment also decreased the SDS resistance and accentuated the effect of dilution (cf. thick bars and Control bars, respectively). Spermatozoa incubated in PF had a much higher resistance to SDS than their controls. Incubation in VF had not such an effect (Fig. 1). This stabilization appeared to be a rapid process, since it became fully developed after only 5 min of "prostatic fluid" exposure in donor B.

2. *Nat'ie SDS sensitivity.* Each of two donors, one with known impaired prostatic function (C) and one with apparently normal prostatic secretion (D) delivered one split ejaculate in two portions (I and II). Aliquots of portion I from both donors were subjected to SDS exposure at 1, 5 and 15 min after

ejaculation (Fig. 2 left: endogenous PF—donor C). The remaining parts of portions I and portions II were centrifuged to obtain PF and the sperm pellets of portions I were used for continued studies. Spermatozoa from donor C, incubated for 5 min in 9 volumes of PF or VF from donor D, and then exposed to SDS (Fig. 2 right: exogenous PF and VF).

The spermatozoa in endogenous (autologous) from donor C showed only a minor increase in SDS resistance from 1 to 15 min after ejaculation. The concentration of zinc and fructose in the were 0.6 and 1.6 mM, respectively.

Spermatozoa from the same PF population (donor C) exposed for 5 min to zinc-rich PF from donor D became almost maximal SDS resistant. Exposure to zinc-poor VF from donor D had no such an effect (Fig. 2 right: PF vs. VF).

Spermatozoa from donor D revealed almost maximal SDS resistance already at 1 min after ejaculation. This initially high SDS resistance was not reduced by subsequent 5 min exposure to zinc-poor PF from donor C. The same result was obtained in 5 additional experiments using 5 different donors where the spermatozoa were exposed

15 min to zinc-poor PF. In the total material the percentage of stable spermatozoa was 93% (range 86-99) before and 94% (84-99) after exposure to the zinc-poor PF.

DISCUSSION

The final steps in the male genome transfer occur in the ovum. Derangement of the sperm head membranes is obligatory (Hiramoto 1962) and occurs concomitant with a rapid decondensation of the clear chromatin (cf. Soupart 1976), ultimately during the male genome available for fusion with the female genome. To obtain fertilizing ability the spermatozoa has to remain in the female genital tract for some hours but the mechanisms involved in the capacitation process are largely unknown (Liu 1951; Chang 1951; Chang & Hunter 1975). Capacitated spermatozoa have an increased metabolism and motility in comparison to the uncapacitated cells but the motility persists considerably longer than the fertilizing ability. The fertilizing ability thus seems to be dependent upon other abilities than those expressed by the increased motility (cf. Chang & Hunter 1975). Capacitation of spermatozoa can also be achieved *in vitro* by exposing them to fluids from the female genital tract. The facilitating factor in bovine follicular fluid has been identified as albumin (Liu, Corbett & Meszel 1977) and albumin is an obligatory component in the fluid used for *in vitro* capacitation (Bavister 1969; Soupart & Morgenstern 1973). Albumin treated human spermatozoa and spermatozoa exposed to human follicular fluid lose about 70-80% of their zinc content (Johansen & Elansson 1976, 1978). Furthermore zinc is removed from rat spermatozoa during their transfer through the female genital tract (Liu & Gould 1958).

Nuclear chromatin decondensation of normal mammalian spermatozoa *in vitro* has earlier been reported by after addition of S-S bond cleaving agents, e.g. DT (cf. Bedford 1975) and it has been suggested that the decondensation *in vitro* is dependent upon S-S cleaving factors in the ovum (Mahl & Yananashi 1975). However the results presented here imply that nuclear chromatin decondensation (NCD) can be elicited also by albumin-facilitated removal of some sperm factor possibly zinc. Thus, addition of albumin to the washing medium conspicuously increased the percentage of spermatozoa undergoing nuclear decondensation in SDS when the albumin treatment was started 15-20 min after ejaculation. From a physiological point of view it seems of interest that one factor (i.e. albumin) known to be responsible for induction of fertilizing ability of ejaculated spermatozoa *in vitro* can release a spermatozoal nuclear chromatin decondensation (NCD) ability that is triggered by SDS derangement of membrane structures. Moreover factors in the seminal plasma present in the 'prostatic fluid' can fully counteract this albumin facilitated NCD-ability.

Thus the studies performed on experimentally SDS sensitized and natively SDS sensitive spermatozoa provide additional and more obvious evidence that a prostatic factor is of major importance for post-ejaculatory inhibition of sperm NCD-ability. Facilitated NCD-ability of spermatozoa from normal donors (induced by albumin or revealed by immediate post-ejaculatory dilution of semen) (see Fig. 1) as well as spontaneous NCD-ability of spermatozoa from a donor with impairment of the prostatic function (Fig. 2) could be inhibited by exposing the spermatozoa to normal prostatic fluid. In contrast normal vesicular fluid did not have this effect.

Indirect evidence that the prostatic factor acts directly upon spermatozoal structures, and not by inhibiting some seminal plasma chromatin decondensing system is provided by the observation that stable spermatozoa did not become SDS sensitized by 15 min exposure to portion I fluid from a donor with impaired prostatic function (Results C 2).

Bedford Bent & Calvin (1973) reported that semen samples from infertile men frequently contained a high percentage of spermatozoa swelling in SDS. This was believed to reflect a dysfunction in the spermatogenesis. The present and previous (Kvist & Elansson 1980; Kvist 1980) results demonstrate the existence of another possibility, i.e. impaired function of the prostate. Thus sperm NCD-ability not counteracted by a prostatic factor at the time of ejaculation may represent an abnormality associated with male infertility.

The events discussed above provides new concepts concerning the physiology of male genome transfer. It is suggested that there exists an intrinsic sperm nuclear chromatin decondensation ability which can be triggered by derangement of sperm nuclear membranes, facilitated by albumin and reversibly inhibited by a factor (zinc) in the prostatic

fluid. Consequently (albumin induced?) removal of spermatozoal zinc in the female genital tract may be of physiological importance as a prerequisite for the final nuclear chromatin decondensation in the ovum.

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Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man

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Nuclear chromatin decondensation (NCD) of ejaculated human spermatozoa was studied in vitro. Spermatozoa subjected to membrane disintegration induced by the detergent sodium dodecyl sulphate (SDS) were found to undergo NCD if previously or afterwards treated with substances known to deplete the spermatozoa of zinc (albumin and EDTA). Zn^{2+} but not other prostatic cations (Ca^{2+} , Mg^{2+}) inhibited the experimentally induced NCD and the NCD of spermatozoa from men with impaired prostatic function. It is suggested that the human spermatozoa has an intrinsic mechanism for NCD that is preserved by temporary zinc inhibition and might be reactivated by zinc removal within the female genital tract.

Key words: Spermatozoa, human, prostatic zinc, EDTA, NCD-ability

vious investigations involving determination of sperm resistance to nuclear swelling in sodium dodecyl sulphate (SDS) suggest that seminal plasma component of prostatic origin plays an important role as a reversible inhibitor of the sperm nuclear chromatin decondensation (NCD) ability (Eklund & Eliasson 1980, Kvist 1980a, b). Recent evidence that the crucial factor might be zinc was provided by the observation that albumin was able to deplete the spermatozoa of zinc continuously reduced sperm resistance to swelling in SDS (Kvist 1980b). Exposure to exogenous Zn^{2+} treatment with albumin, or another zinc-removing substance (EDTA) were used in the present study to provide more direct evidence for the importance of zinc as temporary inhibitor of spermatozoal NCD-ability. Aspects of some of the results presented here were earlier briefly communicated (Eliasson & Kvist 1976, Kvist & Eliasson 1977).

MATERIAL AND METHODS

Semen samples were obtained from men referred to the laboratory for infertility studies and from volunteers (mostly

medical students). All semen samples were collected by masturbation after 3-5 days of continence.

Donors with known impaired prostatic function were chosen to obtain spermatozoa with native sensitivity to react with nuclear swelling in SDS. To obtain semen samples with high (normal) sperm SDS-resistance donors with apparently normal prostatic function were selected (Kvist 1980a). Donors with "apparently normal" prostatic function are defined as men who revealed normal values of the biochemical markers for prostatic secretion in seminal plasma ($Zn > 1.2$ mM, $Mg > 2.9$ mM, acid phosphatase activity > 6.9 U/ml) as well as for seminal vesicular secretion (fructose > 6.7 mM). Donors with impaired prostatic secretion had revealed subnormal seminal plasma concentrations of the prostatic components (cf. Eliasson 1975). Zinc and magnesium concentration in seminal plasma were measured with an atomic absorption spectrophotometer (cf. Eliasson & Lindholmer 1971, 1972) and the acid phosphatase activity according to Sigma Technical Bulletin No 104. Fructose concentration was determined colorimetrically (Karvonen & Mäkelä 1955).

Unwashed spermatozoa were studied in aliquots of whole semen. Washed spermatozoa were prepared by centrifugation of semen (400 g, 15 min) followed by removal of the supernatant. The sperm pellets were thereafter resuspended in a buffered salt solution (BSS) and recentrifuged. The washing procedure was then repeated once. The composition of BSS was (mM) NaCl 123, KCl 5, HCl 14, TRIS 37, pH 8.0. An alternative

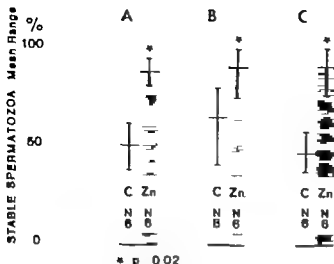


Fig. 1 Zinc-induced normalization of sperm resistance to nuclear swelling in sodium dodecyl sulphate (SDS) of natively SDS sensitive spermatozoa from men with impaired prostatic function. The spermatozoa were exposed to SDS 1 min after zinc treatment. (A) Semen mixed with the equal amount of BSS (C) or BSS containing 3 mM Zn^{2+} (Zn) 2 min after ejaculation. (B) The same procedure as in (A) performed 90 min after ejaculation. (C) SDS resistance of washed spermatozoa without (C) or with the addition of Zn^{2+} (1.6 mM) to the suspension medium.

washing method used to obtain SDS sensitized spermatozoa (Kvist 1980b) was washing in BSS containing 4% albumin (BSSA). When the influence of divalent metallic cations normally present in prostatic fluid (zinc, calcium or magnesium) were studied, water solutions of $ZnSO_4$, $CaCl_2$ were added to BSS in a volume relation of 1:100. In experiments involving sperm treatment with disodium-ethylenediaminetetraacetate (EDTA) a water solution of EDTA was added to BSS or SDS solution in a volume relation of 3:100 providing a final EDTA concentration of 6 mM.

The degree of nuclear chromatin resistance to undergo NCD was assessed as the degree of nuclear swelling after the spermatozoa had been exposed for 60 minutes (when not indicated otherwise) to 1% SDS in 0.05 M borate buffer pH 9.0. One volume of semen or sperm suspension was mixed with 9 volumes of SDS solution and the reaction was stopped by adding the equal amount of 3% glutaraldehyde in 0.05 borate buffer pH 9.0. In 1 experiment the spermatozoa had been pre-exposed to SDS. The NCD was assessed after 60 min incubation in BSS or in BSS containing 6 mM EDTA.

The degree of nuclear swelling was assessed with phase contrast microscopy (500 \times) and the spermatozoa were classified as stable (=not swollen) and unstable (=mode rarely and grossly swollen) (Kvist & Eliasson 1980).

For descriptive statistics mean and range is given for determined percentage of stable spermatozoa and analytical statistics was performed with the Randomization Test for Matched Pairs and the Mann-Whitney U-test for independent groups (cf. Siegel 1946).

RESULTS

Effect of zinc on natively SDS sensitive spermatozoa

Donors ($N=17$) with known impaired prostatic function were selected to obtain semen samples containing spermatozoa with natively low resistance to nuclear swelling in SDS. The effect of exogenous zinc on the SDS resistance of the spermatozoa was studied under three different conditions (series A, B and C).

In series A (6 samples, 4 donors) an aliquot of each semen sample was gently mixed with the equal amount of BSS containing zinc (3 mM) at 1 min after ejaculation. Aliquots mixed with the equal amount of zinc-free BSS served as controls. At 2 min thereafter all specimens were subjected to SDS treatment. As shown in Fig. 1A zinc treatment conspicuously increased the percentage stable (resistant to swelling) spermatozoa (from mean of 51 to $p<0.02$).

The experimental proceeding in series B (6 samples, 5 donors) was the same as in series A with exception that the addition of zinc-containing or zinc-free BSS was not made until approximately 90 minutes after ejaculation. In this case the mean percentage of stable spermatozoa in the control specimens was somewhat higher than in series A. However, also here zinc addition induced a significant increase in the percentage stable spermatozoa (from mean of 65 to 91, $p<0.02$) (Fig. 1B).

The material in series C (6 samples, 4 donors) consisted of spermatozoa washed in BSS approximately 90 min after ejaculation. Aliquots of the sperm population were then resuspended in BSS (controls) or in BSS containing zinc (1.6 mM) and were then within 2 min exposed to SDS. As shown in Fig. 1C zinc treatment of the washed spermatozoa caused an increase in percentage stable spermatozoa ($p<0.02$) from a mean of 47 to 91.

Effect of zinc on experimentally increased sperm SDS sensitivity

(1) *Albumin enhanced sensitivity.* Semen samples ($N=5$) from 5 donors with impaired prostatic function were subjected to BSSA washing 15 min after ejaculation in order to study the effect of the sperm population supersensitized to SDS (Kvist 1980b). After BSSA washing sperm aliquots of each sample were resuspended in pure BSS or BSS containing zinc (1.6 mM) and the sperm

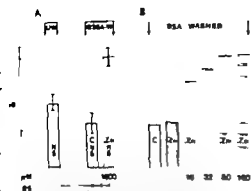


Fig. 2 Effect of zinc on albumin-enhanced sperm nuclear chromatin decondensation in SDS. (A) Spermatozoa from 10th epididymal fraction, UW = Unwashed spermatozoa, BSSA-W = Spermatozoa washed in buffer solution containing albumin, C = Control, Zn = Zinc-treated spermatozoa. (B) Concentration dependent effect of zinc on the sperm resistance to SDS of BSSA-washed spermatozoa.

than 2 min subjected to SDS treatment. For comparison an aliquot of each semen sample (i.e. washed not albumin treated) was subjected to SDS treatment at 15 min after ejaculation.

BSSA-washing increased the percentage of spermatozoa swelling in SDS (Fig. 2A) (C) vs. (UW) ($p < 0.05$). Addition of zinc to the BSSA-washed spermatozoa not only abolished the albumin induced effect, but even induced an SDS-resistance considerably higher than that observed in the unwashed specimens (Zn) vs. (C) and vs. (UW) in Fig. 2A ($p < 0.05$).

In another experiment pooled BSSA-washed spermatozoa were used to study the effect of different concentrations of zinc on the sperm resistance to SDS. Sperm aliquots were resuspended in pure BSS or BSS containing gradually increasing concentration of zinc and were then within 1 min subjected to SDS. As shown in Fig. 2B, zinc concentration of 60 μ M in the medium was sufficient to reverse the proportion stable spermatozoa to over

100% EDTA induced sensitivity. Semen samples from different donors with apparently normal 10th prostatic fraction were used. Within 1 h after ejaculation each semen sample was divided in 3 fractions (I, II, III) in order to determine the preling sperm SDS resistance (fractions I were washed twice in BSS, resuspended in BSS and then immediately subjected to SDS treatment. Fractions

II and III were first washed once in BSS and then in BSS containing EDTA (6 mM). Before SDS treatment fractions II were resuspended in pure BSS whereas fractions III were resuspended in BSS containing zinc (0.16 mM). As shown in Fig. 3A the mean percentage SDS resistant spermatozoa in fractions I was 84. EDTA treatment reduced the percentage stable spermatozoa to 23 (II vs. I in Fig. 3A, $p < 0.05$). However, the addition of zinc to previously EDTA-exposed specimens induced almost full (98%) sperm resistance to SDS (III vs. II and vs. I in Fig. 3A, both differences $p < 0.05$).

The observation that EDTA pre-treatment conspicuously increased sperm sensitivity to subsequent SDS exposure made it interesting to study whether EDTA per se would induce nuclear chromatin decondensation of spermatozoa which previously had been briefly exposed to SDS. Randomly selected semen samples ($N=4$) were pooled and divided in two groups. Both groups were centrifuged and the spermatozoa were resuspended in SDS solution containing zinc (0.16 mM) and there after immediately centrifuged (total time of SDS exposure 10 min). After an intermediate BSS wash

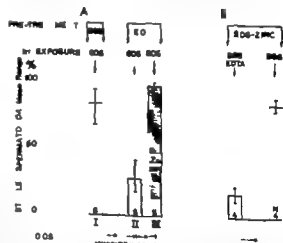


Fig. 3 (A) Effect of zinc on EDTA induced sperm NCD in SDS. I = Spermatozoa washed twice in buffered salt solution (BSS) before SDS exposure, II = BSS-EDTA washed spermatozoa, III = BSS-EDTA washed spermatozoa exposed to Zn^{2+} (0.16 mM) 1 min before SDS treatment. (B) SDS-Zinc pre-treated spermatozoa incubated for 1 h in BSS-EDTA (4%) and in pure BSS (4%). Note that the presence of zinc (0.16 mM) in the SDS-solution did not interact with the SDS dependent part of the NCD process and that the presence of SDS is not needed during the second EDTA-induced part of this process.

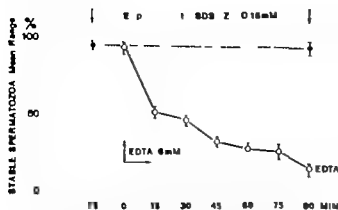


Fig. 4. Dynamics of EDTA-induced NCD studied in 7 groups of washed spermatozoa in SDS containing 0.16 mM Zn (open circles). For comparison is shown the inability of SDS as such to induce NCD (closed circles).

the spermatozoa of one group were resuspended in BSS and those of the other group in BSS containing EDTA (6 mM). Both groups were divided in 4 fractions and stored for one hour before assessment of the degree of nuclear chromatin swelling. As shown in Fig. 3B a high proportion of spermatozoa pre-treated with SDS-Zn remained stable (mean 84% stable spermatozoa) when subsequently incubated in pure BSS whereas most of similarly pre-treated spermatozoa underwent nuclear decondensation when incubated in BSS containing EDTA (mean 27% stable spermatozoa, differences from pure BSS-incubated $p < 0.05$).

Zinc treated spermatozoa were used to study the time dependence of EDTA induced sperm swelling in SDS. The material consisted of randomly selected semen samples ($N=5$) which were pooled and washed in BSS containing zinc (0.16 mM). The spermatozoa were resuspended in SDS solution containing zinc (0.16 mM). The suspension was immediately divided in 9 groups each consisting 4 fractions. To seven of the groups EDTA was added (final concentration 6 mM) after 15 min of SDS exposure and the nuclear swelling reaction was studied in these groups at 15 min intervals from time 0 to 90 min (open circles in Fig. 4). The remaining two groups did not receive EDTA. Here the percentage stable spermatozoa was assessed immediately after SDS exposure and 105 min later (closed circles in Fig. 4). As shown in Fig. 4 addition of EDTA to the SDS-Zn sperm suspensions reduced the percentage stable spermatozoa with time whereas simply SDS-Zn exposure for 105 min did not reduce the percentage stable spermatozoa significantly.

Other prostatic divalent cations and sperm SDS-sensitivity

Spermatozoa BSSA washed 2 h after ejaculate were used to study whether in addition to zinc other divalent cations present in the prostatic fluid (magnesium and calcium) would counteract experimentally induced sperm SDS sensitivity. The material consisted of semen samples ($N=9$) from different donors with apparently normal prostatic function. Each BSSA-washed sample was divided in 4 fractions. One fraction served as control the other 3 fractions either Mg^{2+} , Ca^{2+} or Zn^{2+} added (final concentrations 1.6 mM). The material later all fractions were subjected to SDS treatment. The presence of Mg^{2+} and Ca^{2+} did not change the percentage of SDS resistant spermatozoa over control level (mean and range $Mg^{2+}=69$ (58-78), $Ca^{2+}=69$ (54-78) and controls = 67 (60-77)), whereas the presence of Zn^{2+} in the same concentration induced almost complete sperm SDS resistance (mean and range = 94 (89-97)). (Zn^{2+} treated Mg^{2+} , Ca^{2+} and controls $p < 0.05$).

DISCUSSION

For a successful male genome transfer it is essential that the sperm nuclear chromatin decondenses at the appropriate moment i.e. when the spermatozoon penetrates into the ovum (Barros & Friedlander 1968 cf. Soupart 1976). If for some reason chromatin remains condensed after penetration fertilization is rendered impossible. Two independent pre-requisites are necessary for sperm NCD to occur: disintegration of sperm head membrane (Hiramoto 1962) and cleavage of S-S bridges between chromatin protein subunits (cf. Yanagimachi 1978). During the process of penetration the sperm head membranes disintegrate (cf. Soupart 1976) and it has been postulated that the ovum provides the free thiols necessary for reductive cleavage of S-S bridges in the sperm chromatin (Calvin & Bedford 1971; Mahi & Yanagimachi 1975). The formation of chromatin stabilizing S-S bonds is currently accomplished already during sperm passage through the epididymis (Calvin & Bedford 1971; Bedford, Calvin & Cooper 1973). Recent studies on human spermatozoa (Kvist & Eliasson 1980a, b) have revealed that a factor of prostatic origin efficiently contributes to maintain the stability of condensed sperm nuclear chromatin. The semen from men with impaired prostatic function

found to contain a higher than normal percentage of spermatozoa swelling after membrane disintegration induced by SDS. Furthermore, brief exposure of such SDS sensitive spermatozoa to seminal plasma with normal contribution of prostatic components considerably increased their resistance to SDS. It was also observed that exposure of human spermatozoa to albumin strikingly facilitated NCD during subsequent SDS exposure (Kvist 1980a). Since albumin per se is not likely to induce leakage of chromatin S-S bridges it appeared that the observed effect of albumin was due to removal of some spermatozoal factor maintaining chromatin S-S stability. As albumin previously had been shown to reduce the spermatozoal zinc concentration (Johnsen & Eliasson 1978) it was suggested that removal of intra-cellular zinc in some manner raised NCD after derangement of sperm head structures. It was also postulated that zinc and other origin normally acts to preserve NCD-ability in the appropriate stage of male gamete transfer. It could not be excluded, however, that the observed effect of albumin was due to depletion of some other factor than zinc.

The present study provides more direct experimental evidence for the concept that spermatozoal zinc blocks the NCD-ability of ejaculated spermatozoa until this cation is removed at a later stage of genome transfer. Exposure of human spermatozoa to another substance which causes spermatozoal leakage of divalent cations (EDTA) was found to mimic the effect of albumin (Fig. 3A), and addition of Zn^{2+} to the seminal plasma or to the suspension medium of naturally SDS sensitive spermatozoa (from men with impaired prostatic function) clearly induced a resistance to swelling in SDS of equal magnitude (Fig. 1).

His study also shows that sperm membrane disintegration as such (treatment with the denaturing agent SDS) is not sufficient to induce NCD although it is necessary to reveal sperm NCD ability *in vitro* (Fig. 4). However, a denaturing agent obviously not need to be present any longer during the actual NCD process. Thus, as shown in Fig. 3B, EDTA treatment of spermatozoa previously exposed to SDS-Zn efficiently induced permeability. The experiment illustrated in Fig. 3 also demonstrated that zinc does not interfere with SDS-induced (membrane disintegration) part of the NCD process.

Thiol-induced reductive cleaving of S-S bridges

is regarded obligate for the physiological sperm NCD and it has been suggested that these thiols are provided by the ooplasm (Calvin & Bedford 1971 cf. Yanagimachi 1978). However, NCD was here induced without addition of exogenous thiols. It indicates that NCD was effectuated by thiols "liberated" within the spermatozoon in response to albumin or EDTA treatment and that these thiols upon membrane derangement reductively cleaved the crucial S-S bridges. Consequently the fact that Zn^{2+} counteracted the albumin and EDTA effect suggests that zinc exerted its inhibitory effect on sperm NCD ability by reversibly binding to intracellular thiols. The existence of such a mechanism is also supported by the facts that (a) zinc has a high affinity for thiols (Valle Williams & Coleman 1961) and (b) that removal of zinc is facilitated by thiol reacting compounds in spermatozoa from rats (Calvin & Bleau 1974) and men (Kvist & Eliasson 1978). Spermatozoal free ("unprotected") thiols are readily oxidized in an atmospheric milieu (Marushige & Marushige 1975). However, thiols bound to zinc are considerably less susceptible to oxidation (cf. Chester 1978) and the main physiological importance of spermatozoal zinc might be to protect thiols from oxidative destruction after ejaculation (Kvist 1980c). Subsequent spermatozoal zinc removal within the female genital tract would then unmask thiols making them available for reductive cleaving of chromatin S-S bridges (=reactivated NCD-ability).

The concept of a sperm NCD ability preserved by zinc appears compatible with the facts that (a) the spermatozoon has to remain in the female genital tract for several hours in order to gain fertilizing ability (cf. Chang & Hunter 1975) and it releases zinc during this period (Gunn & Gould 1958), (b) the fertilizing ability is again lost upon sperm re-exposure to seminal plasma making a renewed incubation in the female genital tract necessary to regain fertilizing ability (Chang 1957), (c) fertilizing ability can be induced *in vitro* by sperm exposure to media where albumin seems to be an obligate constituent (Soupart & Morgenstern 1973) and (d) *in vivo* NCD starts concomitant with sperm membrane disintegration, i.e. before the entire sperm head is engulfed by the ooplasm (Barros & Franklin 1968; Bedford 1972; Soupart 1976).

In conclusion, the present and previous studies on human spermatozoa (Kvist & Eliasson 1980a, b, 1980a, b) support the idea that the sper-

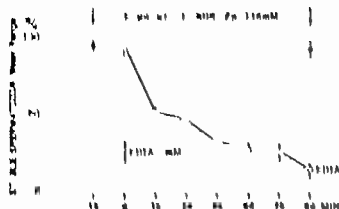


Fig. 4. Dynamic effect of EDTA (added 1 mM) studied in 2 groups of washed spermatozoa in SDS containing 0 (b) and 20 mM Zn²⁺ (a) (b). A comparison is shown the stability of SDS as such is in the NaCl (c) (a) (b) (c).

the spermatozoa of one group was resuspended in BSS and those of the other group in BSS containing EDTA (6 mM). Both groups were divided in 4 fractions and stored for one hour before assessment of the degree of nuclear chromatin swelling. As shown in Fig. 3, a high proportion of spermatozoa pretreated with SDS Zn remain stable (mean 84% stable spermatozoa) when subsequently incubated in pure BSS, whereas most of similarly pretreated spermatozoa and even untreated spermatozoa when incubated in BSS containing EDTA (mean 27% stable spermatozoa) (Differences from pure BSS incubate ($p < 0.05$)).

Zinc free spermatozoa were used to study the time of presence of EDTA in the sperm swimming in SDS. The material consisted of randomly selected semen samples (15–5) which were pooled and washed in BSS containing zinc (0.16 mM). The spermatozoa were resuspended in SDS solution containing zinc (0.16 mM). The suspension was immediately divided in 9 groups each consisting of 4 fractions. In each of the groups EDTA was added (final concentration 6 mM) after 15 min of SDS exposure and the nuclear swelling reaction was studied in these groups at 15 min intervals from time 0 to 90 min (open circles in Fig. 4). The remaining two groups did not receive EDTA. Here the percentage stable spermatozoa was assessed immediately after a SDS exposure and 105 min later (closed circles in Fig. 4). As shown in Fig. 4 and addition of EDTA with SDS Zn sperm suspended in which the percentage stable spermatozoa with time when exposed to SDS Zn exposure for 105 min did not differ from the percentage stable spermatozoa significantly.

Other possible divalent cations and sperm SDS sensitivity

Spermatozoa BSSA washed 2 h after ejaculate was used to study whether in a laboratory other divalent cations present in the products (magnesium and calcium) would counteract eventually induced sperm SDS sensitivity. The total consisted of seven samples (A–G) for 60 sec of incubation with apparently normal preincubation. Each BSSA wash 1 sample was divided in 4 fractions. One fraction served as control, the other 3 fractions with 1 Mg²⁺, 1 Ca²⁺ and 1 of them concentration was 1.6 mM). The other 3 fractions were subjected to SDS test. The presence of Mg²⁺ and Ca²⁺ did not increase percentage of SDS resistant spermatozoa to first level (mean and range: Mg²⁺ 69 (64–74), Ca²⁺ 67 (54–78) and controls 67 (60–74)) as the presence of Zn²⁺ in the same conditions induced almost complete sperm SDS resistance (mean and range: 94 (89–97)) (Zn²⁺ treated: Mg²⁺, Ca²⁺ and controls $p < 0.05$).

DISCUSSION

For a successful maturation of spermatozoa, it is essential that the sperm nuclei chromatin condenses at the appropriate moment (i.e. when the spermatozoa penetrate into the oocyte (Hartley & Liu 1968; cf. Sengpiel 1976)). If this occurs, the chromatin remains condensed after penetration and fertilization is not retarded (Sengpiel 1976). Two factors are required for this process: the spermatozoa must be in a state of chromatin condensation (Hartley 1962) and all steps of SS bonds between chromatin protein subunits (cf. Sengpiel 1976). During the process of penetration, the head membrane distal part is cut (Sengpiel 1976) and it has been postulated that the oocyte penetrates the third is necessary for resins to cross the SS bonds in the sperm chromatin (cf. Hartley 1971; Mahr & Yamashita 1975). The function of chromatin stabilizing SS bonds is eventually accomplished during sperm penetration through the oocyte membrane (cf. Hartley 1976). It is not clear whether the R₁ and R₂ of human spermatozoa (cf. Hartley & Liu 1968; a, b) have also that the R₁ and R₂ of spermatozoa contribute to maintain the integrity of condensed sperm nuclei chromatin. In spermatozoa with impaired penetration, the

found to contain a higher than normal percentage of spermatozoa swelling after membrane integration induced by SDS. Furthermore, brief exposure of such SDS sensitive spermatozoa to seminal plasma with normal contribution of prostatic components considerably increased their resistance to SDS. It was also observed that exposure of spermatozoa to albumin strikingly facilitated NCD during subsequent SDS exposure (Kvist *et al.* 1980b). Since albumin per se is not likely to induce cleavage of chromatin S-S bridges it appeared that the observed effect of albumin was due to action of some spermatozoal factor maintaining chromatin S-S stability. As albumin previously had been shown to reduce the spermatozoal zinc concentration (Uhlenius & Elansson 1978) it was suggested that removal of intra-cellular zinc in some manner preceded NCD after derangement of sperm head structures. It was also postulated that zinc of testis and other origin normally acts to preserve the NCD-ability to the appropriate stage of male meiosis. It could not be excluded however that the observed effect of albumin was due to depletion of some other factor than zinc. The present study provides more direct experimental evidence for the concept that spermatozoal zinc blocks the NCD-ability of ejaculated spermatozoa until this cation is removed as a later stage of genome transfer. Exposure of human spermatozoa to another substance which causes spermatozoal zinc removal (EDTA) was found to block the effect of albumin (Fig. 3A), and addition of Zn^{2+} to the seminal plasma or to the suspension medium of natively SDS sensitive spermatozoa (from men with impaired prostatic function) strongly reduced a resistance to swelling in SDS of similar magnitude (Fig. 1).

The present study also shows that sperm membrane disruption as such (treatment with the denaturing agent SDS) is not sufficient to induce NCD although it is necessary to reveal sperm NCD-ability (Fig. 4). However a denaturing agent obviously not needed to be present any longer during the actual NCD process. Thus, as shown in Fig. 3B, EDTA treatment of spermatozoa previously exposed to SDS- Zn efficiently induced sperm NCD. The experiment illustrated in Fig. 3 also demonstrated that zinc does not interfere with SDS-induced (membrane derangement) part of the NCD process. The fact that thiol-induced reductive cleaving of S-S bridges

is regarded obligate for the physiological sperm NCD and it has been suggested that these thiols are provided by the ooplasm (Calvin & Bedford 1971, cf Yanagimachi 1978). However NCD was here induced without addition of exogenous thiols. It indicates that NCD was effectuated by thiols liberated within the spermatozoa in response to albumin or EDTA treatment and that these thiols upon membrane derangement reductively cleaved the crucial S-S bridges. Consequently the fact that Zn^{2+} counteracted the albumin and EDTA effect suggests that zinc exerted its inhibitory effect on sperm NCD ability by reversibly binding to intra-cellular thiols. The existence of such a mechanism is also supported by the facts that (a) zinc has a high affinity for thiols (Valle Williams & Coleman 1961) and (b) that removal of zinc is facilitated by thiol reacting compounds in spermatozoa from rats (Calvin & Bleau 1974) and men (Kvist & Elansson 1978). Spermatozoal free ("unprotected") thiols are readily oxidized in an atmospheric milieu (Marushige & Marushige 1975). However thiols bound to zinc are considerably less susceptible to oxidation (cf Cheater 1978) and the main physiological importance of spermatozoal zinc might be to protect thiols from oxidative destruction after ejaculation (Kvist 1980c). Subsequent spermatozoal zinc removal within the female genital tract would then unmask thiols making them available for reductive cleaving of chromatin S-S bridges (=reactivated NCD-ability).

The concept of a sperm NCD ability preserved by zinc appears compatible with the facts that (a) the spermatozoa has to remain in the female genital tract for several hours in order to gain fertilizing ability (cf Chang & Hunter 1975) and it releases zinc during this period (Gunn & Gould 1958), (b) the fertilizing ability is again lost upon sperm re-exposure to seminal plasma making a renewed incubation in the female genital tract necessary to regain fertilizing ability (Chang 1957), (c) fertilizing ability can be induced *in vitro* by sperm exposure to media where albumin seems to be an obligate constituent (Soupart & Morgenstern 1973) and (d) *in vivo* NCD starts concomitant with sperm membrane disintegration, i.e. before the entire sperm head is engulfed by the ooplasm (Barros & Franklin 1968, Bedford 1972, Soupart 1976).

In conclusion the present and previous studies on human spermatozoa (Kvist & Elansson 1980, Kvist 1980b) support the idea that the sper-

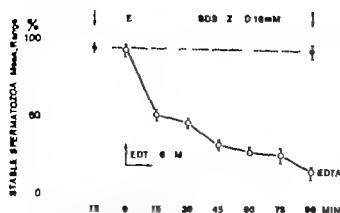


Fig. 4. Dynamics of EDTA-induced NCD studied in 7 groups of washed spermatozoa in SDS containing 0.16 mM Zn (open circles). For comparison is shown the inability of SDS as such to induce NCD (closed circles).

the spermatozoa of one group were resuspended in BSS and those of the other group in BSS containing EDTA (6 mM). Both groups were divided in 4 fractions and stored for one hour before assessment of the degree of nuclear chromatin swelling. As shown in Fig. 3B a high proportion of spermatozoa pre-treated with SDS-Zn remained stable (mean 84% stable spermatozoa) when subsequently incubated in pure BSS whereas most of similarly pre-treated spermatozoa underwent nuclear decondensation when incubated in BSS containing EDTA (mean 27% stable spermatozoa, differences from pure BSS incubated $p < 0.05$).

Zinc treated spermatozoa were used to study the time dependence of EDTA induced sperm swelling in SDS. The material consisted of randomly selected semen samples ($N=5$) which were pooled and washed in BSS containing zinc (0.16 mM). The spermatozoa were resuspended in SDS solution containing zinc (0.16 mM). The suspension was immediately divided in 9 groups each consisting 4 fractions. To seven of the groups EDTA was added (final concentration 6 mM) after 15 min of SDS exposure and the nuclear swelling reaction was studied in these groups at 15 min intervals from time 0 to 90 min (open circles in Fig. 4). The remaining two groups did not receive EDTA. Here the percentage stable spermatozoa was assessed immediately after SDS exposure and 105 min later (closed circles in Fig. 4). As shown in Fig. 4 addition of EDTA to the SDS-Zn sperm suspensions reduced the percentage stable spermatozoa with time whereas simply SDS-Zn exposure for 105 min did not reduce the percentage stable spermatozoa significantly.

Other prostatic divalent cations and sperm SDS sensitivity

Spermatozoa BSSA washed 7 h after epididymus were used to study whether, in addition to zinc, other divalent cations present in the prostatic fluid (magnesium and calcium) would counteract experimentally induced sperm SDS sensitivity. The material consisted of semen samples (A–G) from different donors with apparently normal sperm function. Each BSSA washed sample was divided in 4 fractions. One fraction served as control, the other 3 fractions either Mg^{2+} , Ca^{2+} or Zn^{2+} added (final concentrations 1.6 mM). The material was later all fractions were subjected to SDS treatment. The presence of Mg^{2+} and Ca^{2+} did not elevate the percentage of SDS resistant spermatozoa over control level (mean and range $Mg^{2+}=69$ (54–78), $Ca^{2+}=69$ (54–78) and controls=67 (60–77)), whereas the presence of Zn^{2+} in the same coconcentrated induced almost complete sperm SDS resistance (mean and range =94 (89–97)). (Zn^{2+} treated Mg^{2+} , Ca^{2+} and controls $p < 0.05$).

DISCUSSION

For a successful male genome transfer it is essential that the sperm nuclear chromatin decondenses at the appropriate moment, i.e. when the spermatozoon penetrates into the ovum (Barros & Farrel 1968; cf. Soupart 1976). If for some reason chromatin remains condensed after penetration, fertilization is rendered impossible. Two independent pre-requisites are necessary for sperm NCD to occur: disintegration of sperm head membrane (Hiramoto 1967) and cleavage of S-S bridges between chromatin protein subunits (cf. Yanagimachi 1978). During the process of penetration the head membranes disintegrate (cf. Soupart 1976) and it has been postulated that the ovum provides the free thiols necessary for reductive cleavage of S-S bridges in the sperm chromatin (Calvin & Bedford 1971; Mahi & Yanagimachi 1975). The function of chromatin stabilizing S-S bonds is apparently accomplished already during sperm passage through the epididymis (Calvin & Bedford 1971; Bedford, Calvin & Cooper 1973). Recent studies on human spermatozoa (Kvist & Eliasson 1980a, b) have revealed that a factor of prostatic origin efficiently contributes to maintain the stability of condensed sperm nuclear chromatin. The semen from men with impaired prostatic function

Recovery of choline acetyltransferase activity in the rat urinary bladder deprived of half its innervation

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In the urinary bladder of the rat partially denervated by unilateral removal of the pelvic ganglion 3 days in advance the activity of the acetylcholine-forming enzyme, choline acetyltransferase, measured by a radiolabelling method, was reduced to 58% of the control. A gain in enzyme activity of 28% was found to have occurred when the bladders were examined 25 days postoperatively: the main part of this increase took place during the period 3 to 6 days after the operation and beyond 25 days no further gain in enzyme activity was found. The present findings are compared with previous observations of transient supersensitivity and an increased motor response to electrical stimulation of the intact pelvic nerve of such partially denervated bladder.

Key words. Choline acetyltransferase activity, denervation, nerve sprouting, urinary bladder, rat.

Urinary bladder of the rat receives its cholinergic innervation both from the pelvic and the vagus nerve (see Elmér 1978). On their way to the bladder most hypogastric fibres pass the pelvic plexus (Langworthy 1965; Persson et al. 1973). Activity of the acetylcholine synthesizing enzyme, choline acetyltransferase, has been demonstrated in rat urinary bladder (Ekström 1975), and bilateral removal of the pelvic ganglion reduces the activity of this enzyme to 5% or less of the normal value (Ekström & Elmér 1977). If, on the other hand, the vagus nerve is cut bilaterally only a small reduction of about 16% of short duration is observed, being obvious on the 3rd but not on the 8th day postoperatively. On the 8th day the enzyme activity tended, in fact, to be higher than normal (Ekström & Elmér 1977).

The present study was undertaken to investigate whether rapid restitution in activity of choline acetyltransferase from a low level would occur after lateral removal of the pelvic ganglion. The urinary

bladders were examined 3, 6, 25 and 60 days postoperatively.

A preliminary account of some of the findings has been given (Bannis et al. 1978).

MATERIAL AND METHODS

63 male adult rats of Sprague-Dawley strain bred at the department of Physiology, Lund, were used. At the end of the experimental period they weighed 342 ± 4 g (mean \pm S.E.). Under ether anaesthesia the abdomen was opened and with the aid of dissecting microscope, the left pelvic ganglion with its nerve filaments close to the bladder was removed. The wound was then sutured. The animals were killed by inhalation of ether. The bladders were removed, cleaned and weighed; they were then stored at -20°C .

At each time of observation 8 operated rats and their littermate controls were examined except for the observation at 60 days when the number of operated rats was seven.

In 3 rats both the left and the right pelvic ganglion and their nerve filaments were removed. The bladders of these rats had to be emptied daily by manual pressure under

matozoan has an intrinsic mechanism for NCD that is preserved by temporary zinc inhibition and might be reactivated by zinc removal within the female genital tract.

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der was attempted, the mean bladder weight 178.7 ± 10.8 mg 3 days postoperatively. In one case bladders, the acetylcholine-forming capacity was below the limit of detection. In the others it was 4 and 17 nmol acetylcholine per h respectively.

DISCUSSION

Choline acetyltransferase studied in the present investigation by a radioactive method was probably neuronal origin, since a profound and rapid fall in activity occurred following bilateral removal of pelvic ganglion. The same result has previously been obtained by the use of a bioassay method (Wron & Elmér 1977).

In the urinary bladder partially denervated by total removal of the pelvic ganglion, the acetylcholine acetyltransferase was found to be 58% of control 3 days postoperatively. An increase of 1% from this level of enzyme activity was registered. It was particularly obvious at an early stage between 3 and 6 days and was found to come to an end already when the bladders examined 25 days after the operation. Similar restoration of enzyme activity in the urinary bladder after cutting the hypogastric nerve bilaterally occurred at an early stage (Ekström & Elmér 1977).

It is of interest to compare the findings of the present investigation with those of Ekström, Elmér & Bann (1978) showing that the supersensitivity developed in the urinary bladder to the parasympathetic substance methacholine after removal of the pelvic ganglion on one side was only of a weak nature, being obvious 6–7 days but not 30 days after the operation, and further that the response of the bladder to electrical stimulation of intact pelvic nerve was found to be increased at later observation in spite of the disappearance of sensitization (Ekström & Elmér unpublished observation). The activity of choline acetyltransferase has been shown to be dependent on the flow of nerve impulses (see Ekström 1978). The activity of choline acetyltransferase activity registered in the present study may be a consequence of a compensatory increase in the traffic of motor impulses in the remaining nervous pathway. This effect probably only part of the explanation the appearance of the supersensitivity to metha-

choline and the increased motor responses elicited by nerve stimulation suggest outgrowth of nerves from the intact nervous pathway. This may also be suggested by the finding that the weight of the partially denervated bladders seemed to fall at the later stages of the period of observation, although still being higher than the controls. In the previous work by Ekström, Elmér & Bann (1978) there was no longer any significant difference between the weight of the partially denervated bladder and the control 25–30 days after the operation. A favourable condition for nerve sprouting seems to exist when degenerating and intact nerves intermingle with each other as judged from morphological and functional studies on partially denervated skeletal muscles (see Edds 1953) and sympathetic ganglia (Murray & Thompson 1957). In the urinary bladder such a favourable condition may also occur since the pelvic nerve of each side distributes bilaterally (Carpenter & Rubin 1967; Elmér 1975).

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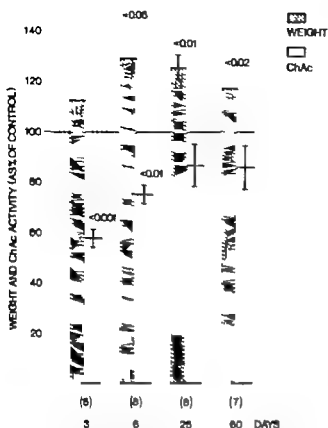


Fig. 1 Weight and choline acetyltransferase activity of urinary bladders at different time intervals after unilateral removal of the pelvic ganglion. The weight and the enzyme activity are expressed as a percentage of those of the urinary bladders of the control litter mate (mean \pm S.E.). Number of comparisons are shown in brackets and when the differences are significant the *P*-values are given.

ether anaesthesia. To avoid bacterial infections 70 mg of a sulfonamide (Sulfuno[®], Nordmark Werke) was given s.c. each day. The animals were killed 3 days postoperatively.

Preparation of the urinary bladder. The bladders were homogenized in 1 ml Na-phosphate buffer 0.1 M pH 6.5 containing dithiothreitol 1.2 mM using a Polytron homogenizer at high speed for 15 s. The homogenate was then usually stored at -20°C for a few days before being analysed.

Estimation of choline acetyltransferase. [Methyl- ^3H] acetyl-CoA was prepared from CoA by treatment with H_2 acetic anhydride (see Hebb et al. 1975). The specific activity measured in a Packard scintillation spectrometer model 3370 (counting efficiency ca. 50%) was 10.4 mCi/mmol.

The medium used for test and control incubation is given in Banns, Ekström & Mann (1979); the control incubation contained acetylcholinesterase instead of eserine. Assays, both test and control, were carried out in duplicate, each tube containing 85 μl of the incubation medium and 25 μl of tissue homogenate. The reaction was started by addition of 10 μl [methyl- ^3H] acetyl-CoA and after 30

min at 39°C it was stopped by addition of 80 μl of chloric acid followed by cooling in an icebath.

Using columns of DeAcidite FFIP resin in the diethyl form [methyl- ^3H] acetyl-CoA was separated from a choline (see Hebb et al. 1975). The true reading for choline formation was obtained by subtracting the m of radioactivity left in the control incubations. Acetylcholine was continually destroyed from the retained in the test incubations where acetylcholine was preserved (see Banns et al. 1979).

The enzyme activity is expressed in nmol acetylcholine produced/bladder h (total activity); the figures are corrected for the volume of the bladders.

Statistics. Student's *t*-test was used; paired comparisons were made between the operated animal and a control litter mate. *P*-values of less than 0.05 were considered significant.

RESULTS

Neither at the start nor at the end of the experiments was there any difference in body-weight between the operated rats and their controls.

The mean weight and the mean choline acetyltransferase activity of the urinary bladders of whole group of 31 control animals was 72.2 ± 11 and 38.7 ± 1.8 nmol acetylcholine formed per bladder per h, respectively.

The weight of the partially denervated bladder was significantly higher than their controls even for the earliest observation, where the difference only tended to be significant ($0.05 < P < 0.1$). When expressed as a percentage the denervated bladder weighed 113 (3 days), 130 (6 days), 176 (25 days), 118 (60 days) % of their respective controls (see Fig. 1).

The activity of choline acetyltransferase in partially denervated urinary bladders was significantly lowered 3 and 6 days postoperatively; it was 58 and 75% of their corresponding control bladders, respectively (see Fig. 1). There was no significant difference between denervated and control bladders 25 and 60 days postoperatively, though the mean percentage figures obtained at these stages were still below the 100% score. On both occasions they were 86.

The choline acetyltransferase activity observed 3 days postoperatively in the partially denervated bladders expressed as a percentage differed significantly from those obtained 6 ($P < 0.05$), 25 ($P < 0.01$) and 60 ($P < 0.01$) days after operation. In 3 rats a complete denervation of the bladder

der was emptied the mean bladder weight was 178 ± 10.8 mg 3 days postoperatively. In one case bladders the acetylcholine-forming capacity was below the limit of detection. In the others it was 4 and 1.7 nmol acetylcholine per h, respectively.

DISCUSSION

Choline acetyltransferase studied in the present investigation by radioactive method was probably of neuronal origin, since a profound and rapid fall in activity occurred following bilateral removal of pelvic ganglion, the same result has previously been obtained by the use of a bioassay method (Bann & Elström 1977).

In the urinary bladder partially denervated by lateral removal of the pelvic ganglion the activity of choline acetyltransferase was found to be 58% in controls 3 days postoperatively. An increase of 28% from this level of enzyme activity was observed. It was particularly obvious at an early stage between 3 and 6 days and was found to come to an end already when the bladders were examined 25 days after the operation. Similar restoration of enzyme activity in the urinary bladder after cutting the hypogastric nerve bilaterally occurred at an early stage (Elström & Elmér 1977).

It is of interest to compare the findings of the present investigation with those of Elström, Elmér & Lönner (1978) showing that the supersensitivity developed in the urinary bladder to the parasympathetic substance methacholine after removal of the pelvic ganglion on one side was only of a transient nature, being obvious 6–7 days but not 40 days after the operation, and further that the response of the bladder to electrical stimulation of the intact pelvic nerve was found to be increased at a later observation in spite of the disappearance of the sensitization (Elström & Elmér unpublished observation). The activity of choline acetyltransferase has been shown to be dependent on the traffic of nerve impulses (see Elström 1978). The recovery in choline acetyltransferase activity reported in the present study may be consequence of a compensatory increase in the traffic of motor impulses in the remaining nervous pathway. This is, however, probably only part of the explanation, the disappearance of the supersensitivity to methacholine

and the increased motor responses elicited by nerve stimulation suggest outgrowth of nerves from the intact nervous pathway; this may also be suggested by the finding that the weight of the partially denervated bladders seemed to fall at the later stages of the period of observation although still being higher than the controls. In the previous work by Elström, Elmér & Bann (1978) there was no longer any significant difference between the weight of the partially denervated bladder and the control 3–30 days after the operation. A favourable condition for nerve sprouting seems to exist when degenerating and intact nerves intermingle with each other as judged from morphological and functional studies on partially denervated skeletal muscles (see Edds 1953) and sympathetic ganglia (Murray & Thompson 1957). In the urinary bladder such a favourable condition may also occur since the pelvic nerve of each side distributes bilaterally (Carpenter & Rubin 1967; Elmér 1975).

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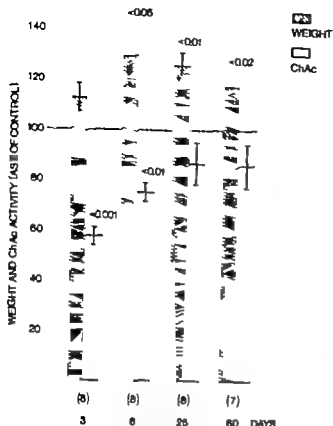


Fig. 1 Weight and choline acetyltransferase activity of urinary bladders at different time intervals after unilateral removal of the pelvic ganglion. The weight and the enzyme activity are expressed as a percentage of those of the urinary bladders of the control litter mate (mean \pm S.E.). Number of comparisons are shown in brackets and when the differences are significant the P values are given.

ether anesthesia. To avoid bacterial infections 20 mg of a sulfonamide (Sulfimo[®], Nordmark Werke) was given s.c. each day. The animals were killed 3 days postoperatively.

Preparation of the urinary bladder. The bladders were homogenized in 1 ml Na-phosphate buffer 0.1 M pH 6.5 containing dithiothreitol 1 mM using a Polytron homogenizer at high speed for 15 s. The homogenate was then usually stored at -20°C for a few days before being analysed.

Estimation of choline acetyltransferase. [Methyl ^3H] acetyl-CoA was prepared from CoA by treatment with H^+ acetic anhydride (see Hebb et al. 1975). The specific activity measured in a Packard scintillation spectrometer model 3320 (counting efficiency ca. 50%) was 10,000 d.p.m./nmole.

The medium used for test and control incubation is given in Bannix, Ekström & Mann (1979). The control incubation contained acetylcholinesterase instead of eserine. Assays, both test and control, were carried out in duplicate, each tube containing 85 μl of the incubation medium and 15 μl of tissue homogenate. The reaction was started by addition of 10 μl [methyl ^3H] acetyl-CoA and after 30

min at 39°C it was stopped by addition of 80 μl of chloric acid followed by cooling in an icebath.

Using columns of DeAcidite FTTP resin in the c form [methyl ^3H] acetyl-CoA was separated from a choline (see Hebb et al. 1975). The true reading for a choline formation was obtained by subtracting the r of radioactivity left in the control incubation. Acetylcholine was continually destroyed from its retained in the test incubations where acetylcholine preserved (see Bannix et al. 1979).

The enzyme activity is expressed in nmol acetylcholine produced/bladder/h. (total activity) the figures are corrected for the volume of the bladders.

Statistics. Student's t -test was used. paired comparisons were made between the operated animal and its control litter mate. P values of less than 0.05 were considered significant.

RESULTS

Neither at the start nor at the end of the experiments was there any difference in body-weight between the operated rats and their controls.

The mean weight and the mean choline acetyltransferase activity of the urinary bladders of the whole group of 31 control animals was 72.2 ± 1.1 and 38.7 ± 1.8 nmol acetylcholine formed per bladder per h respectively.

The weight of the partially denervated bladders was significantly higher than their controls even for the earliest observation, where the difference only tended to be significant ($0.05 < P < 0.1$). Expressed as a percentage the denervated bladders weighed 113 (3 days), 130 (6 days), 176 (25 days), 118 (60 days) % of their respective controls (see Fig. 1).

The activity of choline acetyltransferase in partially denervated urinary bladders was significantly lowered 3 and 6 days postoperatively. It was 58 and 75% of their corresponding control bladders respectively (see Fig. 1). There was no significant difference between denervated and control bladders 25 and 60 days postoperatively, though the mean percentage figures obtained at these stages were still below the 100% score. Both occasions they were 86.

The choline acetyltransferase activity obtained 3 days postoperatively in the partially denervated bladders expressed as a percentage differed significantly from those obtained in controls ($P < 0.02$, $P < 0.01$) and 60 ($P < 0.01$) days after operation. In 3 rats a complete recovery was observed on

Functional and metabolic effects of terbutaline and propranolol in fast and slow contracting skeletal muscle in vitro

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FELLENIUS K., HEDBERG R., HOLMBERG E. & WALDECK B., Functional and metabolic effects of terbutaline and propranolol in fast and slow contracting skeletal muscle in vitro. *Acta Physiol Scand* 1980, 109: 89-95. Received 6 Sept. 1979. ISSN 0001-6772. Research and Development Laboratories, AB Hälsjö Möbels, Department of Zoophysiology, University of Uppsala and Research and Development Laboratories, AB Draco, Lund, Sweden.

The soleus, a slow-contracting, and the extensor digitorum longus (EDL), fast-contracting skeletal muscle from guinea-pig were prepared for isometric recording of sub-tetanic contractions in vitro. The content of adenosine-triphosphate (ATP) and creatinephosphate (CP) together with their metabolites and the contents of lactate, pyruvate and cyclic adenosine monophosphate (cAMP) in the muscles were determined. It was found that the energy and redox state of the isolated soleus and EDL muscles is very stable and does not significantly differ from the normal state in vivo. Moreover there were no consistent changes in these variables after treatment with terbutaline (a β_2 -adrenoceptor agonist) or propranolol or both. Thus, effects on energy metabolism do not seem to cause the changes in muscle contraction, characteristic for β -adrenoceptor stimulation. On the other hand, the functional effects were accompanied by elevation of the cAMP level of the muscles.

Key words: β -adrenoceptor, terbutaline, propranolol, skeletal muscle contraction, energy state, redox state, guinea-pig.

Terbutaline and related compounds have profound effects on the contraction pattern of skeletal muscles.

In slow-contracting muscles the tension and duration of maximal twitches is decreased, resulting in a decrease in the tension and degree of fusion of tetanic contractions. Fast-contracting muscles respond with an increase in these variables (Bowman & Zaiml 1958; Bowman & Nott 1969). These effects on skeletal muscle contraction may have clinical implications. Firstly, during treatment with β -adrenoceptor agonists, muscle tremor appears as an unwanted side-effect (e.g. Thöringer & Sved 1975; Brittain & Jack 1976). This effect may be due to a reduced degree of fusion in slow-contracting muscles (Bowman & Nott 1969). Secondly, treatment with β -adrenoceptor antagonists often gives a sensation of muscle fatigue (Banks et al. 1978; Fellenius et al. 1979). Among several factors it may be the direct cause of muscle weakness,

the importance of cell energy storage compounds has been stressed (Edwards 1978). Lactate accumulation or associated pH changes due to redox changes may be additional factors responsible for the impaired muscle function (Tesch et al. 1978).

Isolated preparations of the slow-contracting soleus and the fast-contracting extensor digitorum longus (EDL) muscles from the guinea-pig have proved useful in the study of the sympathomimetic-induced effects on skeletal muscle contractions (Tashiro 1973; Waldeck 1977; Holmberg & Waldeck 1977; Al-Jeboury & Marshall 1978). Thus the previous in vivo observation that the effects are mediated via β_2 -adrenoceptors (Bowman & Nott 1970; Bohmer & Raper 1976) has been convincingly confirmed. In the present study we have tried to investigate whether the functional effects of terbutaline are

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Functional and metabolic effects of terbutaline and propranolol in fast and slow-contracting skeletal muscle in vitro

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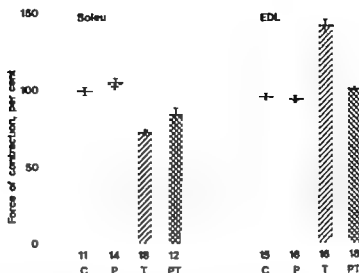


Fig. 1. Effects of propranolol and terbutaline on subtetanic isometric contractions of isolated soleus and EDL muscles. Propranolol (P) ($0.03 \mu\text{mol/l}$) was added to the baths, followed 10 min later by terbutaline (T) ($0.3 \mu\text{mol/l}$). The experiment was stopped after another 20 min. Muscles treated with either drug alone and untreated muscles (C) were run in parallel. The result is expressed in per cent of the force developed by each individual muscle 30 min before the termination of the expt. Means \pm S.E. and at the bottom of each bar the number of expts.

selective β_2 -adrenoceptor agonist (Bergman et al. 1969) are accompanied by changes in energy and redox state. In some experiments propranolol was used to block the effects of terbutaline. The possible role of cAMP as mediator of functional and metabolic effects was also studied. The energy charge and redox state in the isolated in vitro preparation was compared with the corresponding figure in vivo in order to evaluate the viability of the in-vitro preparation.

METHODS

(a) *Animal treatment* Male guinea-pigs weighing about 200 g were used. The soleus and the EDL muscles were dissected out under pentobarbitone anaesthesia and mounted in organ baths containing oxygenated Krebs solution at 37°C . Subtetanic contractions were evoked by transmural field stimulation every 15 s throughout the experiment (Waldeck 1976; Holmberg & Waldeck 1977).

The muscle preparations were allowed to stabilize for 1 h after which propranolol ($0.03 \mu\text{mol/l}$) was added followed 10 min later by terbutaline ($0.3 \mu\text{mol/l}$). After another 20 min the stimulation was stopped and the muscles were quickly removed, wiped off and dropped into liquid nitrogen. Muscles treated with either propranolol or terbutaline and untreated muscles (control) were run in parallel. In some control expts the stimulation was stopped 30 min before the end of the expt. (resting muscles). In order to make comparisons with the metabolic state under in-vivo conditions some muscles were dropped into liquid nitrogen immediately after they had been dissected out. The average weight \pm S.D. was $86 \pm$

20 mg for the soleus and 124 ± 22 mg for the EDL. Muscles were pooled 3 by 3 and stored in liquid air or packed in dry ice until the biochemical analysis.

(b) *Analytical method* The frozen tissue was homogenized and treated with 0.6 mol/l HClO₄ as previously described (Karlsson et al. 1975). The percolate extract obtained was analyzed for adenosine-triphosphate (ATP) (Lamprecht & Trautschold 1962), adenosine diphosphate (ADP) and adenosine-monophosphate (AMP) (Adam 1964), creatinephosphate (CP) (Lamprecht & Stein 1964), creatine (C) (Bernt et al. 1962) and lactate pyruvate (Hohorst et al. 1959). The content of cAMP was analyzed by means of a protein-binding assay (The cAMP Assay, The Radiochemical Centre, Amersham, England, Jan. 1975).

(c) *Statistical analysis* Statistical analysis was formed using analysis of variance followed by the *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

(a) Functional effects

In the soleus muscle terbutaline ($0.3 \mu\text{mol/l}$) depressed the subtetanic contractions by about 25% ($P < 0.001$) (Fig. 1). Propranolol ($0.03 \mu\text{mol/l}$) had no significant effect per se but attenuated the effect of terbutaline ($P < 0.005$). In the EDL the force of subtetanic contractions increased by about 40% at the addition of terbutaline ($P < 0.001$). This effect was completely blocked by propranolol ($P < 0.001$) which by itself was devoid of effect compared with the control expt.

Table 1 The concentration of compounds involved in energy metabolism of the resting and working soleus EDL muscles and the effect of propranolol (0.03 $\mu\text{mol/l}$) and terbutaline (0.3 $\mu\text{mol/l}$) \pm S.E. in $\mu\text{mol/kg}$ wet weight and the number of determinations, each based on three pooled muscles. For further details, see legend to Fig. 1 and the methods section

| | Resting muscles | | Working muscles, in vitro | | | |
|-----|---------------------|---------------------|---------------------------|---------------------|---------------------|---------------------|
| | In vivo | In vitro | Control | Propranolol | Terbutaline | Propr + Terb |
| ATP | $3.3 \pm 0.3 (5)$ | $2.8 \pm 0.2 (4)$ | $2.5 \pm 0.3 (4)$ | $2.5 \pm 0.1 (4)$ | $3.1 \pm 0.2 (4)$ | $2.9 \pm 0.2 (5)$ |
| AMP | $0.73 \pm 0.04 (5)$ | $0.66 \pm 0.03 (4)$ | $0.63 \pm 0.09 (4)$ | $0.55 \pm 0.09 (4)$ | $0.76 \pm 0.05 (4)$ | $0.67 \pm 0.07 (5)$ |
| ADP | $0.07 \pm 0.01 (5)$ | $0.03 \pm 0.01 (4)$ | $0.06 \pm 0.01 (4)$ | $0.02 \pm 0.01 (4)$ | $0.03 \pm 0.01 (4)$ | $0.03 \pm 0.01 (5)$ |
| C | $7.2 \pm 0.6 (5)$ | $8.7 \pm 0.6 (4)$ | $7.2 \pm 1.0 (4)$ | $6.2 \pm 0.4 (4)$ | $7.6 \pm 0.7 (4)$ | $6.5 \pm 1.0 (5)$ |
| CP | $6.2 \pm 0.6 (5)$ | $4 \pm 0.8 (4)$ | $6.9 \pm 0.7 (4)$ | $5.3 \pm 1.6 (4)$ | $5.8 \pm 0.8 (4)$ | $4.7 \pm 0.4 (5)$ |
| ATP | $4.7 \pm 0.3 (5)$ | $3.3 \pm 0.4 (4)$ | $3.9 \pm 0.3 (5)$ | $4.0 \pm 0.1 (5)$ | $3.6 \pm 0.2 (4)$ | $3.8 \pm 0.3 (5)$ |
| AMP | $0.73 \pm 0.01 (5)$ | $0.63 \pm 0.04 (4)$ | $0.59 \pm 0.03 (5)$ | $0.66 \pm 0.03 (5)$ | $0.63 \pm 0.03 (4)$ | $0.55 \pm 0.02 (5)$ |
| ADP | $0.05 \pm 0.01 (5)$ | $0.03 \pm 0.01 (4)$ | $0.03 \pm 0.01 (5)$ | $0.03 \pm 0.01 (5)$ | $0.07 \pm 0.00 (4)$ | $0.02 \pm 0.01 (5)$ |
| C | $11.6 \pm 0.6 (5)$ | $14.1 \pm 0.8 (5)$ | $14.1 \pm 0.4 (5)$ | $13.8 \pm 0.8 (5)$ | $11.1 \pm 0.6 (4)$ | $12.9 \pm 0.8 (5)$ |
| CP | $14.5 \pm 1.1 (5)$ | $7.3 \pm 1.1 (4)$ | $10.9 \pm 1.7 (5)$ | $9.7 \pm 1.0 (5)$ | $10.4 \pm 0.7 (4)$ | $11.3 \pm 2.5 (5)$ |

Effect on the energy state in resting and working control muscle

Results from the determination of ATP and CP together with their metabolites are shown in Table 1. A comparison between resting muscles showed that the level of AMP in the soleus muscle in vitro was half of that in vivo ($P < 0.005$). The same tendency was seen in the EDL muscle ($P < 0.05$). The C level was about 40% lower in vitro than in

vivo ($P < 0.05$). The level of C in EDL in vitro was half that found in vivo ($P < 0.001$) and a similar direction of effect was also seen in the soleus muscle ($P < 0.05$). The product $[\text{ATP}][\text{AMP}][\text{ADP}]$ and $[\text{ATP}][\text{C}][\text{ADP}]$ reflecting the equilibrium state of the adenylate kinase and creatine phosphokinase reaction, respectively tended to be slightly displaced in the direction of ATP-break down in vitro compared with in vivo (Table 1) but

Table 2 The energy state in the resting and working soleus and EDL muscles and the effect of propranolol (0.03 $\mu\text{mol/l}$) and terbutaline (0.3 $\mu\text{mol/l}$) \pm S.E. and the number of determinations, each based on three pooled muscles. For further details, see legend to Fig. 1 and the methods section

| | Resting muscles | | Working muscles, in vitro | | | |
|---|---------------------|---------------------|---------------------------|---------------------|---------------------|---------------------|
| | In vivo | In vitro | Control | Propranolol | Terbutaline | Propr + Terb |
| $\frac{[\text{ATP}]}{[\text{AMP}]}$ | $0.4 \pm 0.04 (5)$ | $0.2 \pm 0.07 (4)$ | $0.4 \pm 0.05 (4)$ | $0.3 \pm 0.05 (4)$ | $0.2 \pm 0.05 (4)$ | $0.2 \pm 0.03 (5)$ |
| $\frac{[\text{ATP}][\text{C}]}{[\text{ADP}]}$ | $3.8 \pm 0.3 (5)$ | $1.2 \pm 0.4 (4)$ | $4.0 \pm 0.3 (4)$ | $3.0 \pm 2.5 (4)$ | $3.2 \pm 0.6 (4)$ | $3.5 \pm 0.5 (5)$ |
| | $0.83 \pm 0.01 (5)$ | $0.88 \pm 0.01 (4)$ | $0.87 \pm 0.01 (4)$ | $0.89 \pm 0.01 (4)$ | $0.88 \pm 0.00 (4)$ | $0.89 \pm 0.00 (5)$ |
| $\frac{[\text{ATP}][\text{AMP}]}{[\text{ADP}]}$ | $0.4 \pm 0.04 (5)$ | $0.2 \pm 0.05 (4)$ | $0.3 \pm 0.11 (5)$ | $0.3 \pm 0.06 (5)$ | $0.2 \pm 0.04 (4)$ | $0.4 \pm 0.21 (5)$ |
| $\frac{[\text{ATP}][\text{C}]}{[\text{ADP}]}$ | $8.1 \pm 1.2 (5)$ | $6.6 \pm 3.6 (4)$ | $5.4 \pm 1.5 (5)$ | $4.5 \pm 0.4 (5)$ | $5.3 \pm 0.3 (4)$ | $6.0 \pm 2.0 (5)$ |
| | $0.91 \pm 0.00 (5)$ | $0.90 \pm 0.01 (4)$ | $0.91 \pm 0.01 (5)$ | $0.91 \pm 0.00 (5)$ | $0.91 \pm 0.01 (4)$ | $0.92 \pm 0.00 (5)$ |

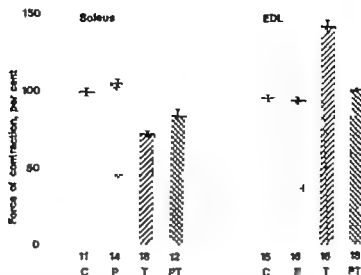


Fig. 1 Effects of propranolol and tertbutaline on subtetanic isometric contractions of isolated soleus and EDL muscles. Propranolol (P $0.03 \mu\text{mol/l}$) was added to the bath, followed 10 min later by tertbutaline (T $0.3 \mu\text{mol/l}$). The experiment was stopped after another 70 min. Muscles treated with either drug alone and untreated muscles (C) were run in parallel. The result is expressed in per cent of the force developed by each individual muscle 30 min before the termination of the exp't. Means \pm S.E. and at the bottom of each bar the number of exp'ts.

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(b) *Analytical methods* The frozen tissue homogenized and treated with 0.6 mol/l HClO₄ as previously described (Harrison et al 1975). The perfusion extract obtained was analyzed for adenosine-triphosphate (ATP) (Lamprecht & Trautschold 1962), adenosine diphosphate (ADP) and adenosine-monophosphate (AMP) (Adam 1962), creatinephosphate (CP) (Larsson & Stern 1962), creatine (C) (Berni et al 1964) and lactate pyruvate (Hohorst et al 1949). The content of cAMP was analyzed by means of a protein-binding assay (The cAMP Assay: The Radiochemical Centre, Amersham, England, Jan. 1975).

(c) *Statistical analysis* Statistical analysis was performed using analysis of variance followed by the *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

(a) Functional effects

In the soleus muscle tertbutaline ($0.3 \mu\text{mol/l}$) depressed the subtetanic contractions by about 15% ($P < 0.001$) (Fig. 1). Propranolol ($0.03 \mu\text{mol/l}$) had no significant effect per se but attenuated the effect of tertbutaline ($P < 0.005$). In the EDL the force of subtetanic contractions increased by about 40% with the addition of tertbutaline ($P < 0.001$). This effect was completely blocked by propranolol ($P < 0.001$) which by itself was devoid of effect compared with the control exp't.

4 The level of c-AMP in the resting and working soleus and EDL muscles and the effect of isobutylmethylxanthine (0.03 $\mu\text{mol/l}$) and terbutaline (0.3 $\mu\text{mol/l}$)

3 E. in $\mu\text{mol/kg}$ wet weight and the number of determinations, each based on 3 pooled muscles. For further see legend to Fig. 1 and the methods section

| Is | Resting muscles | | Working muscles, in vitro | | | |
|----|--|--|--|--|--|--|
| | In vivo | In vitro | Control | Propranolol | Terbutaline | Propr + Terb |
| 1 | 3.7 \pm 1.0 (4) 2.3 \pm 0.2 (5) | 0.50 \pm 0.07 (3) 0.24 \pm 0.02 (3) | 0.85 \pm 0.30 (4) 0.27 \pm 0.03 (5) | 0.49 \pm 0.02 (2) 0.34 \pm 0.03 (4) | 1.22 \pm 0.30 (3) 0.50 \pm 0.01 (4) | 0.81 \pm 0.08 (5) 0.36 \pm 0.05 (5) |

AMP being 7–10 times higher in vivo than in vitro ($P < 0.001$). The level of c-AMP was 2–3 times or in the soleus than in the EDL for the working rats ($P < 0.001$) and for resting muscles (0.025) (Table 4).

Isobutylmethylxanthine doubled the amount of c-AMP in the soleus ($P < 0.01$) and this effect was inhibited by propranolol. The results for the soleus muscle were scattered but muscles treated with terbutaline showed a higher level of c-AMP than those treated with propranolol alone ($P < 0.01$).

DISCUSSION

Findings in this study show that the energy state of the isolated soleus and EDL muscles is very stable and does not significantly differ from the normal state in vivo. This situation is based on the activity of the adenylate kinase and creatine phosphokinase reactions to maintain the involved reactions at concentrations close to equilibrium. For example the mass action ratio of the isolated adenylate kinase (Eggelstone & Hems 1952) is in the range 0.3–0.7. Although the ratios tended to be displaced in the direction of ATP-breakdown in vivo this displacement was close to the normal range. Transmural field stimulation tended to improve the energy state in so far as the mass action ratios were in the direction of ATP-synthesis. The movements may have a beneficial effect on the diffusion of substrates (cf Waldeck 1976). Although muscle movement per se may improve the rate of diffusion of substrates and oxygen, the demand for substrate and oxygen for ATP-synthesis is increased during muscle work. The increase in the acetylpyruvate ratio in the working soleus muscle reflects slightly anaerobic conditions which may be due to a limited supply of oxygen. This effect is probably a normal adaptive phenomenon during

work rather than a difference between in-vivo and in-vitro conditions.

The energy and the redox state of the resting or working muscles in vitro compared with in vivo is thus stable. It may therefore be assumed that the diffusion of substrates and oxygen from the medium is sufficient to maintain a relatively normal metabolism. Similar conclusions have been drawn from experiments on isolated soleus and EDL muscle from the rat (Chandry & Gould 1969; Partington & Wiles 1976).

The effects of terbutaline on subtetanic contraction of the isolated soleus and EDL muscles were similar to those described previously (Waldeck 1977; Holmberg & Waldeck 1977) and they were blocked by propranolol. There were no consistent changes in the energy or the redox state after treatment with terbutaline or propranolol or both. A slight reduction in CP level during terbutaline treatment was however observed in EDL. A similar change in CP level during β -receptor stimulation with adrenaline was previously demonstrated in the vastus muscle of the rat in vivo (Fellenius et al 1979a) without any corresponding changes in the soleus content of CP. This indicates that there may be a quantitative difference in the β -adrenergic control of energy supply in the form of CP in the slow contracting soleus muscle compared with the fast EDL or vastus muscles (cf Fellenius et al 1980). The effect of terbutaline on CP did not significantly influence the energy state. Thus the effects on the energy metabolism do not seem to cause changes in muscle contraction during treatment with β -receptor active compounds (cf Bowman & Nott 1969). No change in CP level was observed in the soleus after drug treatment in spite of pronounced effects on contractility.

The effect of terbutaline on the subtetanic contraction was accompanied by an elevation of the

Table 3 The redox state in the resting and working soleus and EDL muscles and the effect of prop (0.03 $\mu\text{mol/l}$) and terbutaline (0.3 $\mu\text{mol/l}$)

Means in mmol/kg wet weight and the mean ratios, respectively, the \pm S.E. and the number of determinations, *n*, on 3 pooled muscles. For further details, see legend to Fig. 1 and the methods section.

| Muscle and variable | Resting muscles | | Working muscles: in vitro | | | |
|---------------------|---------------------|---------------------|---------------------------|---------------------|---------------------|---------------------|
| | In vivo | In vitro | Control | Propranolol | Terbutaline | Prop + T |
| Soleus | | | | | | |
| Lactate | 1.8 \pm 0.5 (5) | 2.9 \pm 0.3 (4) | 5.3 \pm 0.9 (4) | 4.0 \pm 0.7 (4) | 4.7 \pm 1.0 (4) | 3.4 \pm 0.6 (4) |
| Pyruvate | 0.15 \pm 0.01 (5) | 0.13 \pm 0.02 (4) | 0.13 \pm 0.01 (4) | 0.1 \pm 0.0 (4) | 0.19 \pm 0.04 (4) | 0.11 \pm 0.02 (4) |
| Lact./Pyr | 1 \pm 3 (5) | 23 \pm 4 (4) | 40 \pm 7 (4) | 38 \pm 1 (4) | 26 \pm 3 (4) | 33 \pm 8 (4) |
| EDL | | | | | | |
| Lactate | 4.9 \pm 0.8 (5) | 4.3 \pm 1.0 (4) | 5.6 \pm 0.4 (5) | 5.4 \pm 0.8 (5) | 6.5 \pm 0.6 (4) | 5.0 \pm 0.6 (4) |
| Pyruvate | 0.22 \pm 0.03 (5) | 0.11 \pm 0.01 (4) | 0.1 \pm 0.0 (5) | 0.10 \pm 0.02 (5) | 0.16 \pm 0.0 (4) | 0.14 \pm 0.02 (4) |
| Lact./Pyr | 25 \pm 3 (5) | 41 \pm 10 (4) | 49 \pm 6 (5) | 58 \pm 10 (5) | 43 \pm 9 (4) | 46 \pm 3 (4) |

statistical significance was not attained. The energy charge (EC) reflecting the ratio $[\text{ATP} + 1/2\text{ADP}] / [\text{ATP} + \text{ADP} + \text{AMP}]^{-1}$ (Atkinson 1968) remained very constant in all expts. but it was in general about 2% higher in the EDL than in the soleus muscle ($P < 0.001$). Most of the changes of the compounds involved in energy metabolism seen in the in-vitro preparation were restored to the in vivo levels after transmurial electric stimulation, i.e. in the working muscle. Thus, the increases in AMP ($P < 0.05$) and C ($P < 0.05$) in the soleus muscle during work compared to resting control muscles were statistically significant.

When the soleus muscle was compared with the EDL, e.g. the working control muscles, it appeared that the levels of ATP and CP were about 50% higher in the fast-contracting EDL than in the slow-contracting soleus ($P < 0.001$). The level of AMP in the soleus was twice that found in the EDL ($P < 0.025$) while the level of C showed a difference in the opposite direction ($P < 0.05$).

(c) Effects of terbutaline and propranolol on the energy state

Propranolol and terbutaline, given separately or together, had no significant effects on the levels of ATP or ADP either in the soleus or in the EDL muscle (Table 1). AMP in the soleus, however, was lower in the drug-treated than in the control muscles ($P < 0.05$). The level of CP in the EDL was reduced by about 20% after terbutaline ($P < 0.005$) but no statistically significant changes in CP were induced in the soleus by either propranolol or ter

butaline. Nor did these drugs induce any change in the level of C.

(d) The redox state in resting and working control muscles

In general, the lactate levels tended to be somewhat higher in the EDL compared to the soleus muscle in resting muscles, both in vivo and in vitro (Table 3), although this effect was statistically significant ($P < 0.005$) for the 'in vivo' state only. Since pyruvate did not change significantly, it follows that the lactate/pyruvate ratio was in general higher in the EDL than in the soleus ($P < 0.005$). There was no statistically significant difference in the lactate/pyruvate ratio between the in-vivo and resting in-vitro muscles.

The lactate level in the soleus doubled during work ($P < 0.025$) but no such effect was seen in the EDL. Since pyruvate was relatively constant, the lactate/pyruvate level was increased in the soleus muscle during work.

(e) Effects of terbutaline and propranolol on the redox state

There were no drug-induced effects on the levels of either lactate or pyruvate or on the ratio between the two (Table 3).

(f) Changes in levels of cAMP

Owing to the wide range in the cAMP data, statistical analysis was preceded by logarithmic transformation. The most marked difference observed was that between resting muscles, the

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level of c AMP in the muscle. Accumulation of c AMP after β -adrenoceptor stimulation has previously been demonstrated in both slow and fast contracting skeletal muscle in vivo (Sullivan & Zaimis 1973) and in vitro (Al-Jeboory & Marshall 1978) although there are certain quantitative differences. In our in vivo expts we found a much higher level of c AMP than normal. However, after stabilization in vitro our values averaged 0.5 $\mu\text{mol/kg}$ (cf Steiner et al 1972).

We believe that the stress during the dissection procedure may have caused an increase in c AMP. Al-Jeboory & Marshall (1978) reported a 3 times higher control level. We have no explanation for this difference but it should be noted that in these authors' experiments the concentration of agonist needed to elevate the c AMP level was two orders of magnitude higher than it was in our experiments although they used salbutamol which is more potent than terbutaline (Olsson et al 1979).

In summary, our study lends no support to the view that the functional effects of β -adrenoceptor stimulation are primarily due to changes in the redox and energy state. On the other hand, c AMP appears to be a mediator, as has been proposed by Bowman & Nott (1969 and 1974). Recent studies suggest that secondary to the changes in c AMP there may be changes in the K⁺/Na⁺ balance ultimately leading to changes in the intracellular movement of Ca²⁺ (Holmberg & Waldeck 1980).

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Interaction of Substance P with dispersed pancreatic acinar cells from the guinea pig binding of radiolabelled peptide¹

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SJÖDIN E, BRODIN E, NILSSON G & CONLON T ■ Interaction of substance P (SP) with isolated acinar cells from the guinea-pig pancreas. Binding of radiolabelled peptide. *Acta Physiol Scand* 1980 109-97-105 Received 24 Sept. 1979 ISSN 0001-6772. Section on Pharmacology Department of Drugs, National Board of Health and Welfare, Uppsala, and Department of Pharmacology Karolinska Institute Stockholm, Sweden

Tyr⁸-SP has been radioactively labeled with ¹²⁵I to a specific activity of 200 µCi/µg. This tracer has been found to be rapidly and reversibly bound in a specific way to dispersed pancreatic acinar cells. The specific binding is saturable and dependent on incubation temperature. At 37°C ¹²⁵I-tyr⁸-SP is bound to a few degree sites at lower temperatures about the peptide is rapidly degraded at 37°C. Native SP inhibits binding of tracer. Results have been analyzed according to Scatchard and suggest 2 functionally distinct types of binding sites: a small number of sites with high affinity and a larger number of sites with low affinity for SP. SP also bind cholecystin and physalgin which are peptides similar to SP in terms of chemical structure and biological activities. The concentration range in which SP, physalgin, and cholecystin inhibit binding of ¹²⁵I-tyr⁸-SP correlates well with the range in which these peptides stimulate biochemical processes like outflow of ⁴⁵Ca, accumulation of cyclic GMP and release of amylase from acinar cells.

Key words: Acinar cells, amylase, calcium, cyclic GMP, cholecystin, pancreas, physalgin, substance P

von Euler and Gaddum discovered the presence of a biologically highly active principle in extract from brain and gut that subsequently became known as substance P (SP). Forty years later it was chemically characterized as an undecapeptide (Nilsson et al 1970) and subsequently synthesized (Pear et al 1971). Using radioimmunoassay (SPL) has been demonstrated in extracts of a great number of tissues including the pancreas (Nilsson & Brodin 1971). Immunohistochemical studies have provided additional evidences for the existence of SPL in the nervous structures in the pancreas (Larsson et al 1976, Larsson & Rehfeld 1979). In investigations indicate that exogenously administered SP evokes secretion of amylase from the pancreas (Stark et al 1968, Thulin & Holm 1977). Considering the immunohistochemical and immunological demonstrations of SPL in the pancreas the ability of SP to functionally influence the

exocrine pancreatic cells we decided to investigate whether binding sites capable of specific binding of SP are present on dispersed pancreatic acinar cells from guinea pigs and whether such binding may be correlated to biochemical effects in these cells. The present paper deals with characteristics of binding of SP to dispersed pancreatic acinar cells. In an accompanying paper we study some biochemical effects of SP on acinar cells.

MATERIALS AND METHODS

Male guinea pigs (150-200 g) were obtained from Harry Jansson, Malmö, Sweden. Crude collagenase (E.C. 3.4.24.3) and crude hyaluronidase (E.C. 3.2.1.35) and purified soy-bean trypsin inhibitor were purchased from Sigma Chemical Co. St Louis, Mo. Carrier-free ¹²⁵I (300 µCi/µl) in 0.1 M NaOH was obtained from the

Part of this investigation were presented at XVI Scandinavian Congress of Physiology and Pharmacology, Oslo 1979.

Interaction of Substance P with dispersed pancreatic acinar cells from the guinea pig: binding of radiolabeled peptide¹

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Key words: Acinar cells, amylase, calcium, cyclic GMP, eledoisin, pancreas, physalgin, substance P.

In 1911 von Euler and Gaddum discovered the presence of a biologically highly active principle in extracts from brain and gut that subsequently became known as substance P (SP). Forty years later it was chemically characterized as an endopeptide (Regnier et al. 1970) and subsequently synthesized (Gjell et al. 1971). Using radioimmunoassay (SP-like immunoreactivity (SP-LI)) has been employed to extracts of a great number of tissues including the pancreas (Nilsson & Brodin 1976). Immunohistochemical studies have provided biological evidences for the existence of SP-LI associated to nervous structures in the pancreas (Sjodin et al. 1976, Larsson & Rehfeld 1977). In investigations indicate that exogenously administered SP evokes secretion of amylase from the pancreas (Sjodin et al. 1968, Thulin & Holm 1977). Considering the immunohistochemical and immunological demonstrations of SP-LI in the pancreas the ability of SP to functionally influence the

exocrine pancreatic cells, we decided to investigate whether binding sites capable of specific binding of SP are present on dispersed pancreatic acinar cells from guinea pigs and whether such binding may be correlated to biochemical effects in these cells. The present paper deals with characteristics of binding of SP to dispersed pancreatic acinar cells. In an accompanying paper we study some biochemical effects of SP on acinar cells.

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Radiochemical Centre, Amersham, England. Chloramine T was purchased from Eastman Kodak Co., Rochester, N.Y. sodium metabisulfite from J. T. Baker, Deventer, Holland, bovine serum albumin from Miles Laboratories Inc., Elkhart, Indiana. All other chemicals were of the highest grade commercially available.

Synthetic bovine SP was obtained from Peninsula Laboratories Inc., San Carlos, Calif.

For iodination purposes, an analogue was used in which the phe^4 residue had been substituted by tyr. This analogue was a gift from Dr Karl Folkers, Inst. for Biomed. Res., Univ. of Texas, Austin, Texas. Synthetic bombesin, caerulein, eledoisin and physalaemin were donated by Dr Roberto de Castiglione, Farmitalia S.p.A., Milano, Italy.

Highly purified natural porcine cholecystokinin and natural porcine secretin and natural vasoactive intestinal polypeptide (VIP) were gifts from Dr Viktor Mutt, GIH research unit, Karolinska Institutet, Stockholm, Sweden.

Highly purified natural porcine gastric inhibitory polypeptide (GIP) was donated by Dr John C. Brown, Dept. of Physiology, Univ. of British Columbia, Vancouver, B.C.

Preparation of ^{125}I -tyr-SP (labelled SP)

Tyr-SP was iodinated according to the chloramine T method (Hunter & Greenwood 1962). The procedure was performed at room temperature in a 1×70 mm polystyrene tube. $300 \mu\text{Ci Na}^{125}\text{I}$ in $20 \mu\text{l}$ of 0.5 M sodium phosphate buffer, pH 7.5, was reacted with $3 \mu\text{g}$ (3.7 nmol) Tyr-SP dissolved in $50 \mu\text{l}$ 0.15 M sodium chloride by addition of $10 \mu\text{g}$ (35.5 nmol) of chloramine T in a volume of $10 \mu\text{l}$. After 30 s the reaction was terminated by addition of $20 \mu\text{g}$ (105.2 nmol) of metabisulfite dissolved in a volume of $20 \mu\text{l}$. (Both the chloramine T and the metabisulfite were dissolved in 0.25 M phosphate buffer, pH 7.5.) The reaction volume was then diluted with 1.0 ml 0.15 M sodium chloride containing 50 mg (740 nmol) of bovine serum albumin and transferred to another test-tube containing 10 mg of microfine precipitated silica (Quosol 3, Philadelphia Quartz Co., Philadelphia). Labelled SP was bound to the silica and later eluted with a mixture of acetone and acetic acid as described by Yalow & Berson (1966) for parathyroid hormone.

After this preliminary purification the iodinated material was subjected to gel filtration on Sephadex G 25 superfine ($9 \times 600 \text{ mm}$) at 4°C using 0.05 M veronal buffer, pH 8.6 with 4 mg (59 nmol) of bovine serum albumin per ml as eluent. Fractions (0.85 ml) were collected and counted directly in a gamma counter. The fractions giving the highest degree of binding in our RIA system (Nilsson et al. 1977) were pooled and used for the receptor-binding experiments. The specific activity of the ^{125}I -tyr-SP as calculated by comparing self-displacement curves of tracer with ordinary standard curves in the RIA system was $200 \mu\text{Ci}/\mu\text{g}$.

Preparation of dispersed pancreatic acinar cells

Guinea pigs were deprived of food overnight and killed by a blow to the head. The pancreas was dissected free from fat and mesentery and dispersed acinar cells were prepared as described by Gardner et al. (1975) using minor modifications of the technique of Am. terdam & Jamieson

(1972, 1974). Cells were suspended in Krebs-Ringer bicarbonate (pH 7.4) equilibrated with 95% O_2 and 5% CO_2 containing L-amino acid supplement (Eagle 1959), soybean trypsin inhibitor per ml 15 mM glucose (w/v) bovine serum albumin and 0.5 mM calcium. Cell concentration was $12 \times 10^6 \pm 0.8 \times 10^6$ cells/ml.

Binding assay

Cells were incubated with ^{125}I -tyr-SP at a concentration of $5 \times 10^{-10} \text{ M}$ at various temperatures ($4, 12, 22$ and 37°C). At appropriate times after addition of ^{125}I -tyr-SP, $100 \mu\text{l}$ or triplicate $100 \mu\text{l}$ samples were layered over $100 \mu\text{l}$ Krebs-Ringer bicarbonate buffer in microfuge tubes polypropylene. The cells were centrifuged at $10400 \times g$ in a Beckman model B microfuge. The pellet was washed a total of 4 times. The microfuge tubes or washed pellets were placed in a Packard 4600 gamma counter. Duplicate samples were also taken from the incubation mixture containing both cells and acinar cells placed in the counter.

The obtained values were used to calculate total ^{125}I -tyr-SP as the percentage of added ^{125}I -tyr-SP. Binding of radioactive SP in the presence of 10^{-6} M cell referred to as nonspecific binding.

This value was on average equal to $38 \pm 3\%$ of the total binding observed at equilibrium conditions.

Dissociation of bound labelled SP

Cells were preincubated with ^{125}I -tyr-SP for 10 min at 22°C as described above (Binding assay). After centrifugation (2 min , $500 \times g$) the supernatant was aspirated and cells resuspended in fresh buffer with or without labelled SP (10^{-6} M) and a second incubation started at 4 or 37°C . Samples were taken from the incubation mixture immediately and after $10, 20, 30, 45$ and 60 min and binding was determined. Total radioactivity remaining in medium after resuspension was less than 3% of the initial value.

Functional alteration (deteriorated binding) of labelled SP following incubation with acinar cells

^{125}I -tyr-SP was incubated with cells for various times at different temperatures (30 min at 37°C , 60 min at 22°C and 90 min at 4°C). Cells were then centrifuged for 10 min at $10000 \times g$. The supernatant was aspirated and added to fresh cells in such numbers that concentration remained unchanged. Binding of labelled SP to cells was then determined at 22°C as described (Binding assay) after various incubation times and expressed as percent of maximal uptake of labelled SP in incubations in which fresh tracer had been added to

Chemical alteration (chromatographic behaviour) of labelled SP following incubation with acinar cells

^{125}I -tyr-SP was incubated in buffer solution with or without cells at different temperatures ($1, 22$ and 37°C) as described above and samples ($300 \mu\text{l}$) taken from incubation mixture at various times ($1, 10, 30 \text{ min}$). Samples were spun (15 s) in a microfuge, the supernatants removed and placed in plastic tubes and then immediately into an acetone-dry ice bath. This procedure requires

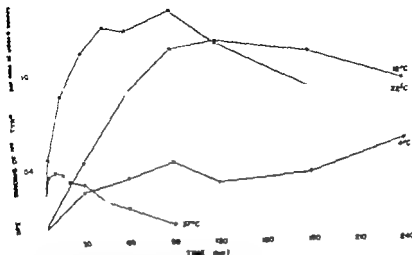


Fig. 1 Time course of specific binding of ^{125}I -tyr⁸ SP to pancreatic acinar cells as a function of incubation temperature. Cells were incubated with $5 \cdot 10^{-8}$ M ^{125}I -tyr⁸ SP. Each point represents the mean of results from 4–6 xpts.

imately 1 min. Samples were then stored at -20°C and chromatographed on Sephadex G-25 superfine column using the same parameters as described under Preincubation of ^{125}I -tyr⁸ SP. The extent of degradation was assessed from the elution curves of radioactivity and to which the samples were compared control of labelled SP taken directly from storage and diluted to similar concentration and volume as the incubation samples before being chromatographed on the same column.

ULTS

Time course of specific binding of labelled SP to dispersed pancreatic acinar cells (Fig. 1)

At a temperature of 37°C specific binding of ^{125}I -SP was maximal after 10 min incubation and then decreased gradually to 10% of the maximal value after 90 min. At 22°C binding was as rapid initially as at 37°C . However, during incubation for 45 min binding increased further after which it levelled off at a degree of 1.3% specific binding.

After 90 min of incubation at this temperature specific binding tended to slowly decrease. A reduction of the incubation temperature to 12°C decreased the binding rate but the maximal specific binding was not significantly different from that observed at 22°C . Incubation at 4°C further reduced the rate of binding and the highest degree of specific binding obtained during 4 hours of incubation was at this temperature around 0.6%.

When the pH of the incubation medium was

varied within the range of pH 6.6–8.4 binding was found to be maximal at pH 7.8. Results are not shown.

B. Time course of dissociation of bound labelled SP (Fig. 2)

The dissociation rate was found to be temperature dependent over the temperature range tested (12 – 37°C) being highest at 37°C . At 22°C the initial rate was approximately 2%/min. Addition of nonradioactive bovine synthetic SP (10^{-6} M) did not affect the rate of dissociation at any temperature.

C. Functional alteration (deteriorated binding) of labelled SP following incubation with acinar cells (Fig. 3)

The low degree of specific binding of ^{125}I -tyr⁸ SP at 37°C suggested to us that SP was rapidly broken down in our system at that temperature. To study the functional alteration of SP during incubation with acinar cells, binding experiments were performed after preincubation of ^{125}I -tyr⁸ SP with cells at different temperatures and for various periods of times (see methods). In all cases preincubated radioligand gave lower maximal specific binding compared to what was obtained with fresh labelled SP. The impairment of binding was however much more pronounced following incubation at 37°C than at 22° or 12°C , maximal binding being 30%, 50% and 58% of maximal binding of fresh

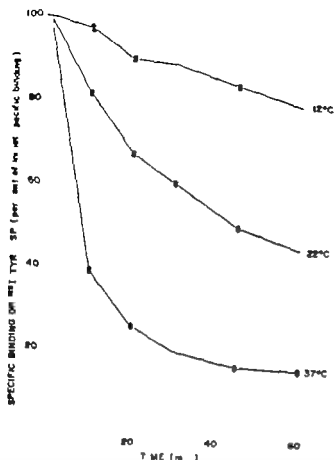


Fig 2 Effects of temperature, dilution and dilution with addition of native SP (10^{-8} M) on dissociation of specifically bound 125 I-tyr 8 -SP from pancreatic acinar cells. Cells were preincubated with 125 I-tyr 8 -SP (5×10^{-6} M) for 60 min at 22°C. Cells were then spun at $500 \times g$ for 2 min. Supernatant was aspirated and cells resuspended in fresh buffer of indicated temperature with and without native SP (10^{-8} M). Graph shows the percentage of 125 I-tyr 8 -SP specifically bound to the cells immediately after resuspension which remains on the cells at indicated times thereafter. Each point represents the mean of 3 separate expts. Closed circles (●) denote resuspension in buffer alone and open circles (○) denote resuspension in buffer with 10^{-8} M native SP.

tracer respectively in spite of shorter preincubation times at the higher temperatures.

D Chemical alteration (chromatographical behaviour) of labelled SP following incubation with cells (Figs 4 and 5)

In order to further study the possible enzymatic degradation of 125 I-tyr 8 -SP during incubation, buffer solutions were incubated with and without cells at different temperatures and for various times. The degree of chemical alteration of the tracer was then estimated from the elution patterns of radioactivity following gel filtration on Sephadex G-25 superfine. The fraction of radioactivity eluting in the peak

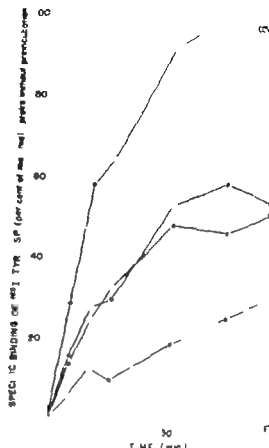


Fig 3 Determination of bound 125 I-tyr 8 -SP after preincubation at various temperatures with pancreatic acinar cells. Cells were incubated for 30 min at 37°C, 60 min at 22°C and 90 min at 12°C with 125 I-tyr 8 -SP (5×10^{-6} M). Cells were then spun in a microfuge for 15 s (1000 $\times g$). The supernatant was aspirated and added to fresh buffer whose uptake of tracer was determined at 22°C at indicated times. Results are expressed as percent of net uptake of 125 I-tyr 8 -SP in control incubations in which tracer had been added to cells. Each point represents mean of 3-4 expts.

corresponding to intact labelled SP was moderately reduced with increasing incubation time at 12 and 22°C while incubation at 37°C produced a shift of radioactivity to the right in the elution pattern indicating a rapid destruction of labelled SP at this temperature. Thus the results of the control experiments showed the same general tendency; the results of the rebinding experiments demonstrating a clear temperature dependence of degradation rate.

E Inhibition of binding of labelled SP by synthetic SP and structurally related peptides (Fig 6)

The ability of various concentrations of synthetic SP, edetolisin and physalgin to inhibit binding

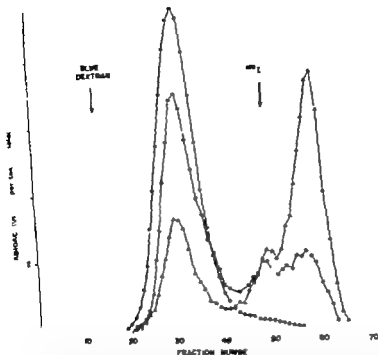


Fig. 4. Determination of free ^{125}I -tyr⁸ SP. Cells were incubated with ^{125}I -tyr⁸ SP for various times at different temperatures. A 300 μl sample was taken and spun in the microfuge (10 000 $\times g$) for 15 s. The supernatant was aspirated and stored at -20°C until put on Sephadex G-25 superfine column (9 \times 600 mm) and run with veronal buffer. The graph shows the elution patterns for an unincubated standard preparation of ^{125}I -tyr⁸ SP (closed circles) and the supernatant from cell suspension incubated with ^{125}I -tyr⁸ SP for 1 h at 12°C (open circles) or 37°C (triangles).

^{125}I SP on acinar cells was examined. Incubations with synthetic SP were carried out at 22°C for 5 or at 12°C for 90 min. The results were usually the same at both temperatures. Inhibition of specific binding of labelled SP could be detected at 1×10^{-10} M. SP was halfmaximal at 10^{-9} M, bound 70% at 2×10^{-9} M. At 3×10^{-9} M specific binding was about 5% of control binding. The data were also plotted according to Scatchard (1949). The plot displayed an upward concavity and the first part of the curve could be described by 2 pH lines. This suggests to us that there may be distinct types of binding sites for SP on the acinar cells. We have calculated the apparent affinity and capacity for each type from results of 11 rate experiments at 22°C and 5 experiments at 12°C . Similar results were obtained for incubation temperatures indicating that there are approximately 300 high affinity sites and 2000

sites of the low affinity type per cell. Half of the high affinity sites will be occupied at a SP-concentration of 9×10^{-10} M and 50% of the low-affinity sites at about $2-3 \times 10^{-9}$ M. These figures must be interpreted with caution, since our experimental conditions do not meet all criteria required for this type of analysis (Rodbard 1973; Boeynaems & Dumont 1975). Eledoisin and physalaemin which have structural similarities with SP also inhibited binding of ^{125}I -tyr⁸ SP to the acinar cells. Only relatively high concentrations of eledoisin and physalaemin (10^{-6} M) did inhibit binding.

Some other peptides that have earlier been shown to have biological effects on pancreatic acinar cells were also tested for ability to interfere with binding. However, secretin at a concentration of 5×10^{-6} M, GIP at 1×10^{-6} M, caerulein, bombesin and VIP at 10^{-6} M did not significantly affect binding of ^{125}I -tyr⁸ SP.

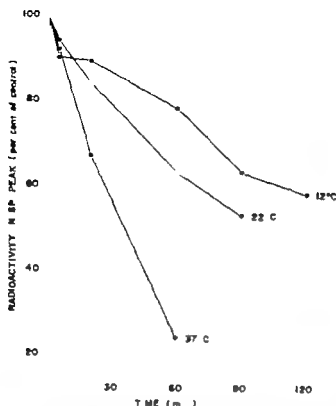


Fig. 5 Deterioration of free $[^{125}\text{I}]$ -tyr⁸ SP. Cells were incubated with $[^{125}\text{I}]$ -tyr⁸ SP for indicated times at indicated temperatures. A 300 μl sample was taken and spun in the microfuge (10 000 $\times g$) for 15 s. The supernatant was aspirated and stored in a deepfreeze until put on a Sephadex G-25 superfine column (9 \times 600 mm) and run with veronal buffer. The graph shows percent of radioactivity remaining in initial peak representing intact $[^{125}\text{I}]$ -tyr⁸-SP. In control experiments with incubation of $[^{125}\text{I}]$ -tyr⁸-SP in buffer without cells there was no significant degradation of SP at 12°C and 22°C while there was approximately 20% degradation at 37°C after 1 h.

DISCUSSION

Specific binding of radioactively labelled SP or tyr⁸ SP has earlier been demonstrated to a crude synaptic membrane preparation from rabbit brain (Nakata et al. 1978) and to isolated synaptic vesicles from rat brain (Mayer et al. 1979).

The present paper demonstrates binding of labelled SP to pancreatic acinar cells and further illustrates that the binding is rapid, specific, reversible and dependent on incubation temperature. The relatively low degree of maximal specific binding obtained at 37°C incubation temperature compared to what was obtained at lower temperatures seems to be explained by a SP degrading activity present in the pancreatic acinar cells. Both the functional and chemical alteration of labelled SP following in

Table 1 Characteristics of binding sites for tyr⁸ SP on dispersed guinea-pig pancreatic cells

$S_{0.5}$ is concentration (M) of SP required to occupy the indicated type of $[^{125}\text{I}]$ -tyr⁸ SP binding sites. The represents means \pm S.E. from 10 separate experiments and 5 expts. at 12°C.

| | High affinity SP binding site | Low affinity SP binding site |
|--------------------------|--|--|
| Number of sites per cell | 260 ± 50^{27} 360 ± 60^{12} | 2300 ± 150^{27} 1400 ± 90^{12} |
| $S_{0.5}$ | $8.8 (\pm 1.5) \times 10^{-12}$ ²⁷ $9.5 (\pm 1.0) \times 10^{-12}$ ¹² | $3.1 (\pm 0.4) \times 10^{-11}$ ²⁷ $2.1 (\pm 0.7) \times 10^{-11}$ ¹² |

cubation with cells were studied and indeed a clear temperature dependence was observed, speaking in favour of an enzymatic nature of the degrading activity. The occurrence of SP-degrading enzymes has formerly been shown in organs and tissues including for example (Benuck & Marks 1975) kidney (Ward & Johnson 1978) endothelial cells (Johnson & Erdös 1977) blood (Bury & Mashford 1977). In an attempt to inhibit the SP inactivation during the experiments aprotinin (Trasylo[®] 500 kIU/ml) bacitracin (50 $\mu\text{g}/\text{ml}$) were included in the reaction medium in some experiments. Aprotinin and bacitracin in these concentrations have in other systems been shown to inhibit SP-degradation (Johnson & Erdös 1977; Cuello et al. 1977; Beck et al. 1978). In our hands these measures were ineffective.

Scatchard plots of $[^{125}\text{I}]$ -tyr⁸ SP binding at 12°C and 22°C were curvilinear with an upward concavity. The curves could be fitted to two straight lines. This has been interpreted as indicating that there were two functionally distinct classes of SP binding sites. Since the rate of dissociation of $[^{125}\text{I}]$ -tyr⁸ SP was not altered by addition of cells, our data do not appear to demonstrate non-cooperativity (Kahn 1975; Boeynaems & Quidley 1975). We do not know if the two types of binding sites exist on all cells or if there are two populations of cells with different types of binding sites.

Physalaemin and eledoisin are two peptide hormones with chemical and biological characteristics similar to SP, which have been isolated from the skin of a fish (physalaemin) and from the posterior pituitary gland of a Mediterranean octopus (eledoisin).

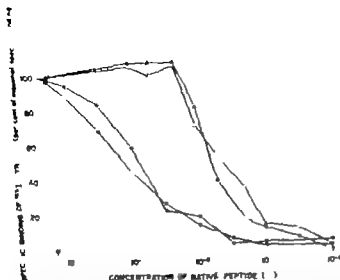


Fig. 6. Ability of native SP, elodeisin, and physalaemin to inhibit binding of ^{125}I -tyr⁸-SP (5×10^{-10} M) to pancreatic acinar cells. Cells were incubated with native SP either for 60 min at 22°C (open circles) or 60 or 90 min at 12°C (closed circles). In experiments with elodeisin or physalaemin incubation were carried out at 22°C for 60 min. Open triangles denote experiments with physalaemin and filled triangles experiments with elodeisin. Binding of ^{125}I -tyr⁸-SP is expressed as percentage of radioactivity bound specifically in the absence of added nonradioactive peptide. Each point represents the mean of at least 6 separate expts.

ner group (Amastasi & Erspamer 1960, Ieraciotti et al. 1965).

Isolated pancreatic acinar cells have been shown to incubate with physalaemin and in with elevated calcium outflow, increased accumulation of cyclic GMP and increased release of amylase (May III et al. 1978). Our present results show that binding sites for SP on the acinar cells also bind these two peptides. The reported data for elodeisin and physalaemin also correlate with reported biochemical effects of these peptides on acinar cells (May III et al. 1978). Thus it has been found that elodeisin and physalaemin in a concentration of 3×10^{-6} M significantly stimulate the accumulation of cyclic GMP. This combination of the peptides produced 60–70% inhibition of ^{125}I -tyr⁸-SP binding.

In some preliminary experiments we have observed inhibition of ^{125}I -tyr⁸-SP by addition of unlabeled tyr⁸-SP instead of native SP. In contrast to Sjödén et al. (1979) who found that tyr⁸-SP did not interfere with binding of ^{125}I -tyr⁸-SP to synaptic vesicles we have found that tyr⁸-SP inhibits binding of ^{125}I -tyr⁸-SP to pancreatic acinar cells to a degree similar to that obtained with native SP.

The results of our study of the biochemical effects of SP on calcium outflow from acinar cells, cellular accumulation of cyclic GMP and stimulation of amylase release are reported as an accompanying paper. Effects on these parameters are seen with concentrations of SP which are in the same range as those which inhibit binding of ^{125}I -tyr⁸-SP. Maximal effects on cyclic GMP accumulation and amylase release are seen at a concentration of 3×10^{-6} M SP (Sjödén et al. 1979).

Excellent technical and secretarial assistance by Carin Gustavsson, Kristina Uddhoken, Ylva Ekblom, Eva-Lis Pettersson and Elisbeth Lindberg is hereby acknowledged. This investigation was financially supported from Swedish Medical Research Council (K5205, K5211) the Swedish Society of Medical Sciences, Roth and Richard Julem fund, Åke Wibergs stiftelse and Harald and Oreta Jeansson stiftelse.

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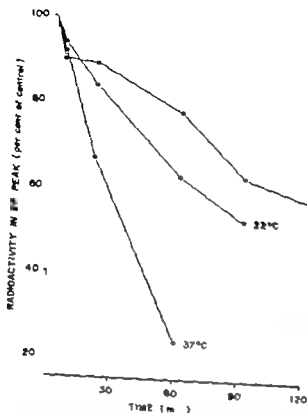


Fig. 5 Deterioration of free ^{125}I -tyr-SP. Cells were incubated with ^{125}I -tyr-SP for indicated times at indicated temperatures. A 300 μl sample was taken and spun in the microfuge (10000 \times g) for 15 s. The supernatant was separated and stored in a deepfreeze until put on a Sephadex G-25 superfine column (9 \times 600 mm) and run with veronal buffer. The graph shows percent of radioactivity remaining in initial peak representing intact ^{125}I -tyr-SP. In control experiments with incubation of ^{125}I -tyr-SP in buffer without cells there was no significant degradation of SP at 12°C and 22°C while there was approximately 20% degradation at 37°C after 1 h.

DISCUSSION

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| | High affinity SP binding site | Low affinity SP binding site |
|--------------------------|--|--|
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| S_0 | $8.8 (\pm 1.5) \times 10^{-12} \text{ M}^{*2}$ $9.5 (\pm 1.0) \times 10^{-12} \text{ M}^{*2}$ | $3.1 (\pm 0.6) \times 10^{-11} \text{ M}^{*2}$ $1.1 (\pm 0.7) \times 10^{-11} \text{ M}^{*2}$ |

cubation with cells were studied and to obtain a clear temperature dependence was obtained speaking in favour of an enzymatic nature of degrading activity. The occurrence of SP-degrading enzymes has formerly been shown in organs and tissues including for example (Benuck & Marks 1975) kidney (Ward & L 1978) endothelial cells (Johnson & Erdös 1977) blood (Bury & Mashford 1977). In an attempt to inhibit the SP inactivation during the experiments aprotinin (Trasylo[®] 500 kIEU/bactracin (50 $\mu\text{g}/\text{ml}$) were included in the reaction medium in some experiments. Aprotinin and bactracin in these concentrations have not been shown to inhibit SP-degradation (Johnson & Erdös 1977; Cuervo et al 1977; Beck et al 1978). In our hands these measures were ineffective.

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Interaction of Substance P with dispersed pancreatic acinar cells in the guinea pig. Stimulation of calcium outflux and accumulation of cyclic GMP and amylase release

SJÖDIN THOMAS P CONLON CARIN GUSTAVSON and KRISTINA UDDHOLM

SJÖDIN L., CONLON T P, GUSTAVSON C. & UDDHOLM, K.. Interaction of Substance P (SP) with dispersed pancreatic acinar cells from the guinea pig. Stimulation of calcium outflux, accumulation of cyclic GMP and amylase release. *Acta Physiol Scand* 1980, 109: 107-110. Received 24 Sept. 1979. ISSN 0001-6772. Section on Pharmacology, Department of Drugs, National Board of Health and Welfare, Uppsala, and Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden.

In dispersed acinar cells from the guinea pig pancreas, substance P (SP) was found to stimulate outflux of ^{45}Ca , cellular accumulation of cyclic GMP and release of amylase. Maximal effects on accumulation of cyclic GMP and release of amylase were obtained with $3 \cdot 10^{-6}$ M of SP. 10^{-6} M of SP caused maximal outflux of ^{45}Ca . These effects corresponded to 30-50% of the maximal effects obtained with caerulein, a cholecystokinin-like decapeptide. The concentrations of SP required for stimulation of ^{45}Ca outflux, accumulation of cyclic GMP and release of amylase corresponded well with those which affect binding of ^{35}S -labeled SP to pancreatic acinar cells.

Key words: Acinar cells, amylase, caerulein, calcium, cyclic GMP, pancreas, substance P

As shown in an accompanying paper that substance P (SP), a radioactively labelled derivative of SP, is specifically bound to dispersed pancreatic acinar cells from the guinea pig. Others have shown that substance P in high concentrations cause cyclic GMP concentrations in pancreatic acinar cells from the guinea pig (Albano Bhoola & Hey 1978). Major stimulants of enzyme secretion from the pancreas like cholecystokinin, and alcohol have been thought to act upon the acinar cells through stimulation of calcium outflux and accumulation of cyclic GMP (Gardner et al. 1976; Sjödin & Gardner 1977). SP has also been reported to stimulate pancreatic enzyme secretion in vivo (Starks et al. 1968; Lin & Holm 1977). We have investigated the effects of SP on calcium outflux, accumulation of cyclic GMP and amylase release from dispersed acinar cells from the guinea pig pancreas and extended the correlation between these effects of SP and binding of SP to the cells. We have compared effects of SP with those of a cholecystokinin-peptide, caerulein.

MATERIALS AND METHODS

In addition to the peptides and reagents listed in the preceding paper (Sjödin et al. in press) ^{45}Ca (12.5 μCi per mg), ^{35}S -succinyl GMP TME antigen, ^{35}S -succinyl cyclic AMP TME antigen and cyclic GMP and AMP specific radioassays were purchased from New England Nuclear Corp., Boston, Mass. Hebes (N-2-hydroxyethyl-piperazine-N'-ethanesulfonic acid) from Kabi, Stockholm and Phadebas amylase test tablets from Pharmacia Liochemicals AB, Uppsala, Sweden.

Dispersed pancreatic acinar cells were prepared according to the technique of Amsterdam and Jamieson (1972, 1974). Unless otherwise specified cells ($12 \cdot 10^6 \pm 0.8 \cdot 10^6$ cells/ml) were suspended in a standard incubation solution composed of Krebs-Ringer bicarbonate (pH 7.4) with L-amino acid supplement (Eagle 1959), 0.1 mg of soybean trypsin inhibitor per ml, 15 mM glucose, 1.8% (w/v) albumin and 0.5 mM calcium, and gassed with 95% O_2 , 5% CO_2 .

Outflux of ^{45}Ca . Cellular loss of ^{45}Ca was measured as described by Gardner et al. (1975). Cells were preincubated with ^{45}Ca for 60 min and the loss of cellular radioactive

Parts of this investigation were presented at XVI Scandinavian Congress of Physiology and Pharmacology, Oulu 1979.

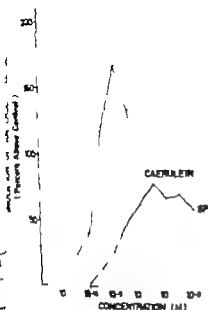


Fig. 3. Effect of caerulein and substance P on release of amylase from dispersed pancreatic acinar cells. Acinar cells were suspended in standard incubation solution in the presence of N -[2-hydroxy-5-ethylpiperazine- N' -ethanesulfonyl]-L-glutamic acid (TEPES) replaced bicarbonate and which contained 1 mM calcium and 5 mM theophylline. Release of amylase was calculated as the difference in the percentage of amylase released at the beginning and end of the 30-min incubation at each concentration. Each point represents the mean of at least 18 incubations.

Fig. 3). Increase in maximal release of amylase by SP was approximately 30% of the increase induced by a maximal dose of caerulein (Fig. 3).

DISCUSSION

Cholecystokinin-related substances and cholinergic agents (Christophe et al. 1971; Sjödén & Gardner 1977). SP appears to stimulate amylase release from pancreatic acinar cells by activation of calcium influx and accumulation of cyclic GMP. The efficacy of SP was relatively low. For calcium influx, cellular accumulation of cyclic GMP and amylase release, maximal effects obtained with SP were 30–40% of those with caerulein. The relative insensitivity of SP on different parameters involved in acinar cell secretion suggests that they act through different biochemical pathways. An alternative way of stimulation of pancreatic enzyme secretion may be through accumulation of cyclic AMP. Amylase

release induced by secretin and vasoactive intestinal polypeptide (VIP) appears to be mediated through cyclic AMP (Gardner, Conlon & Adams 1976; Robberecht, Conlon & Gardner 1976; Gardner & Jackson 1977). However, neither SP in the present study nor cholecystokinin (Gardner, Conlon & Adams 1976) stimulate cyclic AMP accumulation in acinar cells.

Our findings in the accompanying paper dealing with binding of 125 I-tyr⁸-SP to acinar cells (Sjödén et al. in press) suggest that caerulein does not interact with the receptor for SP. Earlier findings indicate that cholecystokinin-like peptides and cholinergic agents act through different receptors on acinar cells (Gardner et al. 1975; Christophe et al., 1976). A third type of membrane receptors on acinar cells, specific for bombesin (Eisensamer & Melchiorri 1975) has recently been reported (Jensen et al. 1978). Bombesin also increases calcium outflux, cyclic GMP and amylase secretion (May et al. 1978). However, bombesin according to our results (Sjödén et al. in press) does not interfere with binding of 125 I-tyr⁸-SP. The present study thus indicates that there is a fourth type of receptors affecting the same system. Our accompanying paper suggests that there is a high affinity and a low affinity binding site for SP. Occupation of the high affinity binding sites apparently contributes to a substantial extent to the subsequent biochemical effects.

May and co-workers (1978) found that the SP-like peptides eledoisin and physalaemin stimulated calcium outflux, cellular cyclic GMP accumulation and amylase release. Since these peptides have affinity towards the SP binding sites, it is reasonable to assume that they exert their effects on outflux of calcium, accumulation of cyclic GMP and release of amylase by occupation of the same receptors as SP. This hypothesis is also supported by the observation that maximal effects on these parameters appear to be the same with SP, eledoisin and physalaemin.

To what extent SP plays a role in the physiological regulation of pancreatic exocrine secretion cannot be decided from the present experiments. Demonstrations of SP-like immunoreactivity in the pancreas (Larsson, Sundler & Håkansson 1976; Larsson & Rehfeld 1979) and stimulatory effects of SP on pancreatic enzyme secretion during *in vitro* (Albano, Bhoola & Harvey 1977) and *in vivo* conditions (Starke et al. 1968; Thulin & Holm 1977)

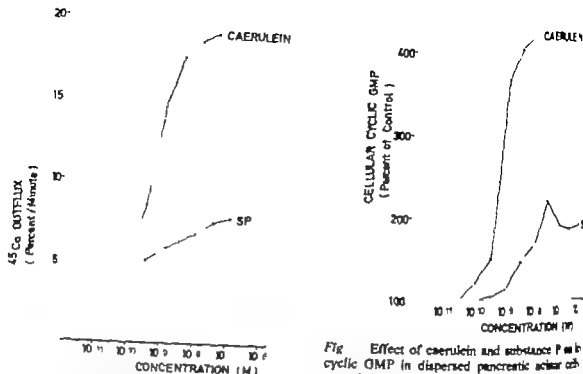


Fig. 1 Effect of caerulein and substance P on ^{45}Ca outflux from dispersed pancreatic acinar cells. Acinar cells were preincubated in standard incubation solution containing $0.5 \text{ mM } ^{45}\text{Ca}$ for 60 min at 37°C . Appropriate concentrations of the indicated agents were added and ^{45}Ca outflux was calculated from the loss of the cellular ^{45}Ca during a 5-min incubation with 5 mM ethylenediaminetetraacetate (EDTA). Each point represents the mean of at least 11 separate incubations.

Fig. Effect of caerulein and substance P on cellular cyclic GMP in dispersed pancreatic acinar cells. Cells were incubated at 37°C in standard incubation solution containing 0.5 mM calcium and 5 mM theophylline. Cellular cyclic GMP was determined at the end of incubation with caerulein or 1 min with SP. Each point represents the mean of at least 4 separate experiments.

tivity during a 5 min incubation period at 37°C with 5 mM ethylenediaminetetraacetate (EDTA) was determined.

Cellular cyclic GMP and AMP. Cellular cyclic GMP and AMP levels were measured by radioimmunoassay. Acinar cells were suspended in standard incubation solution containing 5 mM theophylline and incubated at 37°C . Incubations were stopped by adding ice ethanol. The samples were centrifuged at $1000 \times g$ for 20 min. An aliquot of supernatant was acetylated by addition of triethylamine and acetic anhydride and then assayed for cyclic GMP or AMP as described by Harper & Brooker with some modifications (1975). Standards were prepared by adding known amounts of cyclic GMP or AMP to standard incubation solution containing 5 mM theophylline and processed the same way as the experimental samples.

Amylase release. For amylase release experiments, acinar cells were suspended in buffer identical to the standard incubation solution except that the bicarbonate was replaced by an equimolar amount of HEPES (pH 7.4) and that it contained 5 mM theophylline. Gassing was performed with $100\% \text{ O}_2$. After 0 and 30 min of incubation at 37°C an aliquot of cell suspension was centrifuged at $10000 \times g$ for 15 s using a Beckman Microfuge (Beckman Instruments, Inc. Spinco Division, Palo Alto, Calif.). An adequately diluted aliquot of supernatant was assayed for

amylase activity by the method of Ceska, Brubaker & (1969) using the Phadebas reagent as substrate. To measure total amylase present in the suspension, an aliquot of cell suspension was added to 10 volumes of a solution containing 0.01 M sodium phosphate (pH 7.5), 0.1% albumin and 0.1% (w/v) sodium dodecylsulfate and assayed for amylase activity. The rate of release was taken as the difference between the amylase present in the supernatant at the start of and after 30 min of incubation and was expressed as the percentage of amylase activity.

RESULTS

Incubations with increasing concentrations of caerulein induced dose related enhancement of ^{45}Ca outflux from acinar cells (Fig. 1). $3 \times 10^{-10} \text{ M}$ caerulein produced a slight stimulation of calcium outflux. At a concentration of 10^{-7} M the rate of ^{45}Ca outflux was only about 40% of that attained with $3 \times 10^{-8} \text{ M}$ of caerulein (Fig. 1).

$3 \times 10^{-10} \text{ M}$ of SP increased cellular accumulation of cyclic GMP to a maximum of about 40% of that obtained with 10^{-8} M caerulein (Fig. 2). Neither caerulein nor substance P had any significant effects on AMP levels (not shown).

Amylase release was augmented by SP. It reached a maximum at a concentration of $3 \times 10^{-10} \text{ M}$.

Method to Improve the size uniformity of microspheres

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microspheres are used to measure blood flow the amount that may be injected is limited by factors, primarily a rise in arterial pressure. It is therefore desirable that the proportion of spheres actually get stuck in the capillaries is as large as possible. Too small spheres, which pass through capillaries, as well as too large spheres, which are held up in the arterioles, should be avoided.

especially the case if blood flow is measured in tissue segments, since the spheres found there so few that the statistical variability of the result becomes embarrassing. From this point of view commercially available microspheres, in particular the more expensive varieties, tend to have wide size ranges. Also in developing automatic devices for sphere counting uniform sphere size is advantageous.

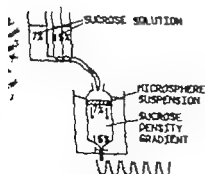
Therefore, a procedure to separate a batch of spheres into fractions with better size definition may be useful. Källskog et al. (1977) published such a method where microspheres were allowed to sediment in water. Here a modification of this method is presented (Fig. 1). The essentials are that aggregation of the spheres is combined with dextran and Tween, and that convec-

tion is diminished with the aid of a sucrose density gradient. In this way fractions with a standard deviation of size of approx. 1 μm may be achieved.

Materials and methods 10 μm microspheres produced by New England Nuclear were used. These spheres are relatively inexpensive and do not aggregate much, but vary much in size (Fig. 2).

The separation was carried out in Macrodex (Pharmacia) with 0.01% Tween-80 added. Macrodex, being a medical product, is expensive but easily available. Any similar dextran preparation should be as useful.

Solutions of sucrose, 7% and 15% in Macrodex-Tween were prepared and allowed to reach room temperature. With 200 ml each of these solutions, using standard equipment (two communicating vessels) a sucrose density gradient was laid in a 500 ml cylindrical separating funnel with 6 cm diameter kept thermostated in a water bath close to room temperature. The system is not extremely temperature-sensitive, and in a room where sudden draughts etc. can be eliminated, the water bath may not be necessary. Laying the gradient took about 1 h, and a further 30 min were allowed for stabilization.



The experimental apparatus employed. The water surrounds the funnel where the separation takes place. The equipment at the left is used to make the sucrose density gradient.

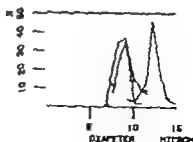


Fig. 2 The result of successful separation. Crosses show the composition of the original batch. Dots show the composition of 3 fractions. Distributions are founded on samples of size 100.

have suggested a physiological role for SP in the regulation of pancreatic exocrine secretion. The present study has shown specific binding sites for SP on pancreatic acinar cells and demonstrated a correlation between occupation of SP-binding sites and activation of amylase secretion. According to Larsson & Rehfeld (1979) most SP in the pancreas is located in peptidergic nerves which mainly innervate ganglia. They suggest that the ganglia represent an important physiological site of action in the pancreas for SP. The present results show that SP also acts directly on the acinar cells of the guinea pig.

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Demonstration of a proton gradient across the insulin granule membrane

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In the last few years the existence of a proton-transporting mechanism in the chromaffin granule membrane has been well established (Johnson et al. 1978). Proton translocation enables the granules to maintain an internal pH below that of the surrounding cytoplasm. One probable physiological function of the electrochemical gradient is to provide energy for the accumulation of amines within granules (Johnson et al. 1978a). This mechanism for accumulation of amines in granules is a general phenomenon. Johnson et al. (1978b) demonstrated the presence of a pH difference in the serotonin granules of platelets and the ability to concentrate amines in secretory granules shared by all cells producing polypeptide hormones (Owman et al. 1973). In the latter types of cells the primary function of the proton gradient differs from that of genuine amine secreting cells. In the pancreatic β -cell the stored insulin is extremely sensitive to pH, exhibiting maximal activity at pH 6.0 (Coore et al. 1969), whereas the cellular pH has been estimated to be as high as 7.2 (Hedman et al. 1972). It has also been suggested that uptake of Ca^{2+} into the insulin-containing β -granules occurs in exchange for protons (Rasmussen et al. 1979). So far there has been no demonstration of ΔpH in isolated β -cells or granules from any other polypeptide hormone-producing cell. By using a highly sensitive fluorimetric technique (Salama et al. 1980) it has been possible to perform such measurements on minute amounts of granules that can be obtained from isolated pancreatic islets.

Four β -cell-rich pancreatic islets were collagenase-isolated from ob/ob mice starved overnight. A secretory granule fraction was prepared by repeated centrifugation at pH 7.0 as previously described (Kobner et al. 1979). The granule pellet suspended in 10 μl 0.25 M sucrose buffered at pH 7.0 with 10 mM Tris-maleate. The suspension kept on ice until use. A Aminco-Bowman

spectrofluorometer equipped with a 3 \times 3 mm microcuvette was used to measure pH-dependent fluorescence changes of 9-aminoacridine. The monochromators were set for narrow-band excitation and emission at 400 and 435 nm respectively. Fluorescence changes were recorded on a potentiometric recorder.

Each experiment was started by the addition of 90 μl of the Tris-maleate buffered sucrose medium supplemented with 11 μM 9-aminoacridine to the cuvette. After 1 min, 10 μl of the granule suspension was mixed with the contents of the cuvette. The addition of the granules prompted a gradual decrease of the fluorescence signal which reached a steady level within 30 s (Fig. 1). Subsequent mixing with 1 μl 4 M NH_4Cl gave a rapid increase of the fluorescence approaching that of 10 μM 9-aminoacridine (the final concentration). The quenching of 9-aminoacridine fluorescence obtained with different granule fractions varied more than could be expected from their protein content. It was checked that the fluorescence was not significantly affected by light scattering from the granules and that the effect of NH_4Cl could not be attributed to a direct interaction with 9-aminoacridine itself.

The present technique is based on the ability of amines such as 9-aminoacridine with relatively high pK_a values to permeate freely biological membranes only in their neutral form. The distribution of the protonated form will therefore depend only on ΔpH (Johnson et al. 1978; Salama et al. 1980). It is assumed that the fluorescence from 9-aminoacridine taken up by the granules is completely quenched (Salama et al. 1980).

In the particular experiment shown in Fig. 1 the addition of β -granules to the 9-aminoacridine medium was associated with a fluorescence decrease more than 3 fold greater than that expected from dilution alone. The kinetics of this decrease was compatible with intragranular accumulation of the amine. After addition of excess NH_4^+ to short

200 mg spheres were suspended in 70 ml of an equal mixture of Macrodex Tween and distilled water. Denser suspensions were difficult to apply without convection. The suspension was thermostated in the same water bath. When the density gradient was ready, the suspension was thoroughly stirred and carefully spread on top of the sucrose solution with the aid of a syringe. 2–3 l were allowed for sedimentation; thereafter the contents of the funnel were withdrawn in 6 fractions.

Results. The method is somewhat delicate in that small mistakes may cause convection, yielding in

ferior separation. In a successful run, most of the microspheres were contained in fractions with a standard deviation of diameter of about 1% or less (Fig. 7). The rest of the spheres could be collected with little loss and rerun.

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are hypoglycemia leads to accumulation rachidonic acid in brain tissue

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ary of Lund, Sweden

ow well known that brain tissues only contain
ll pool of free fatty acid (FFA), of which the
ne arachidonic and docosahexaenoic acids
re a minor part (see Bazin 1976, Marion &
1979). This pool increases rapidly in ischemia
der those circumstances, the polyenoic
orm the major part of the pool. Accordingly
een concluded that ischemia preferentially
es phospholipase A₂.

discovery that arachidonic acid is the sub-
of fatty acid cyclooxygenase and thereby the
rior of prostaglandins and related substances,
d to a surge of interest in the FFA metabolism
in tissues. Thus, since the cyclo-oxygenase
is substrate-dependent, accumulation of
idonic acid may lead to a burst of synthesis of
aglandins and related substances. There is a
ng suspicion that the cascade of events thus
d contributes to reversible and irreversible
damage (see Alarcon & Garbus 1975
1979).

activation of phospholipase A is not well
lsood but it has been speculated that it occurs
relations of cellular energy failure with release

• (Alarcon & Garbus 1975, Flower &

• well 1976). Apart from ischemia, seizures in-

• l by electroshock or pentylenetetrazole ad-

• mination have been found to elevate brain tissue

• tration of arachidonic acid (Bazin 1976).

• errably respiratory distress in animals with

• res could have contributed to cerebral energy

• l this report we give a preliminary account

• with demonstrating a marked increase in brain

• arachidonic acid concentration during severe

• glycemia, a condition leading to ATP depletion

• and accumulation of tissue oxygen supply

• tional. The experiments were performed on

• Wistar rats (230-430 g) that were fasted for 4

• d then injected with insulin (40 U.L.) \times kg \times
• at 30 min after the injection the animals were

anesthetized with 3% halothane tracheotomized,
mmobilized with tubocurarine chloride (0.5 mg \times
kg) and artificially ventilated on about 1%
halothane, 75% N₂O and 25% O₂. Femoral arterial
and venous catheters were inserted for blood pres-
sure recording, sampling of blood, and i.v. injec-
tions. Electrodes were inserted into the skull bone
for EEG recordings, and provision were made for
freezing the brain *in situ* with liquid nitrogen. Ven-
tilation was then continued on 75% N₂O and 25% N₂.
Body temperature was maintained at 37°C, arterial
PCO₂ at 35-40 mmHg and arterial PO₂ at 100
mmHg or higher. Unoperated control animals were
maintained under similar anesthesia conditions for
comparable periods.

Following freezing of the brain *in situ* and stor-
age at -80°C, cortical tissue was dissected at -20°C
and extracted with chloroform-methanol. After
centrifugation and washing the FFA pool of the
extract was separated by thin layer chromatography
and the free fatty acids were determined with gas-
liquid chromatography. A full account of the
techniques is given elsewhere (Rehoczka et al.
1980).

Results and Discussion Following insulin injec-
tion blood glucose concentration gradually de-
creases and when it falls below about 1 μ mol \times g
spontaneous EEG activity ceases (see Agardh et al.
1978). In the present series brains were sampled for
analyses 5, 15, 30 and 60 min following cessation of
EEG activity.

In control animals the FFA concentration of the
cerebral cortex was $0.117 \pm 0.007 \mu$ mol \times g (mean
 \pm S.E., $n=6$). After 5 min of hypoglycemic coma
the value increased to $0.751 \pm 0.065 \mu$ mol \times g. The
FFA pool then remained virtually constant at 15, 30
and 60 min of coma. Fig. 1 shows values for palmitic
(16:0), stearic (18:0) and arachidonic (20:4)
acid. Results on the first two of these acids were
typical of all others measured except one. Thus the

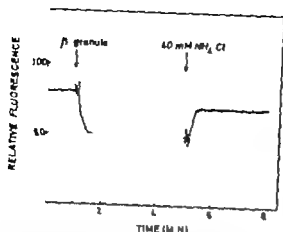


Fig. 1 Demonstration of a Δ pH in a β -granule fraction by measurements of the fluorescence quenching of 9-aminoacridine. The cuvette initially contained 90 μ l 0.5 M sucrose, 10 mM Tris-maleate (pH 7.0) and 11.1 μ M 9-aminoacridine. The fluorescence was quenched by the addition of 10 μ l of a β -granule suspension and was enhanced by obliterating the Δ pH by addition of NH_4Cl to a final concentration of 40 mM.

circuit the proposed proton gradient there was a rapid release of the amine.

When intact pancreatic islets are exposed to 5-hydroxytryptamine or its precursor 5-hydroxytryptophan there is a rapid accumulation of large amounts of the amine within the β -granules (Hellman et al 1977b; Gylfe 1978). However as indicated by efflux studies (Gylfe 1978) the amine is not firmly bound within the β -cells. Furthermore inhibition of the β -cell metabolism resulted in a rapid depletion of the amine (Gylfe 1980). Since the maintenance of a proton asymmetry in the β -granules would be energy-dependent the latter experimental situation is probably analogous to the addition of NH_4^+ . In summary the present experiments support the existence of a proton gradient in the β -granules which might account for the accumulation of different amines in these organelles.

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Inhibition of fast anterograde axoplasmic transport by a pressure barrier. The effect of pressure gradient and maximal pressure

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HAHNENBERGER, L. P. W. Inhibition of fast anterograde axoplasmic transport by a pressure barrier. The effect of pressure gradient and maximal pressure. *Acta Physiol Scand* 1980; 109: 117-121. Received 23 March 1979. ISSN 0001-6772. Department of Medical Pharmacology and Ophthalmology, University of Uppsala, Sweden.

Fast anterograde axoplasmic transport was studied in the motor fibres of the vagus nerve of rabbits *in vitro* at 22°C with a pressure barrier applied to a small section of the nerve. The barrier was characterized by the maximal pressure and the gradients on each side of the maximum. The ranges tested were 16 to 45 mmHg maximal pressure and 30 to 140 mmHg/mm length of nerve for the pressure gradients. It was found that in the ranges tested the maximal pressure was of major importance for the inhibition of transport, the pressure gradient being of little significance. The results indicate that at least one part of fast anterograde axoplasmic transport occurs within collapsible canalicular structures.

Key words: rabbit, vagus, axoplasmic transport, pressure barrier, maximal pressure, pressure gradient, endoplasmic reticulum, sliding filament.

In recent years there has been considerable progress in the characterization and understanding of the role of axoplasmic transport (for a review see Rafferty 1977). However, our knowledge of the mechanism of this intracellular transport system is fragmentary. As far as is known, different components are transported anterogradely at at least different rates: one slow and one fast (Griffin, Karlsson & Sjöstrand 1969). This transport depends on local energy supply (Ochs 1971b) and is inhibited by various agents (Pamison & McEwen 1972; Edström & Hansson 1975) of which the microtubules are of major importance (Dahlsström). Several mechanisms have been proposed for axoplasmic transport. Basically there are two main hypotheses which may be called the streaming process and the canalicular models.

Edström (1968) first suggested that components migrate along the outer surface of microtubules. This idea was developed in more detail by Ochs (1971). He introduced the hypothesis that long filament loaded with material is transported along the microtubules. Fast axonal transport should thus depend on the presence of intact microtubules. The disruption of their structure by

antimitotics (Shefanski 1972) which also inhibit axoplasmic transport (Dahlsström 1968) is thus consistent with the "sliding filament" hypothesis. The

canalicular model on the other hand postulates that an axoplasmic movement takes place within canalicular structures. Palay (1958) first pointed out, that the channels of the endoplasmic reticulum might be a possible pathway. These structures, like the microtubules, run longitudinally along the axon from the perikaryon to the synaptic region (Droz et al. 1975; Tsukita & Ishiawa 1976). Support for this view was later provided by radioautographic studies by Droz and his group (Droz 1975; Droz et al. 1975) and others (Schönbach et al. 1971).

In a recent paper a method was described by which it is possible to compress a section of a nerve *in vitro* with a well defined, adjustable and stable dynamic pressure barrier without interfering with the supply of nutrients and oxygen (Hahnenberger 1978).

The purpose of the present study was to attempt to assess the two models by a physiological approach, based on the following idea: if material moves along a nerve by any mechanism the move-

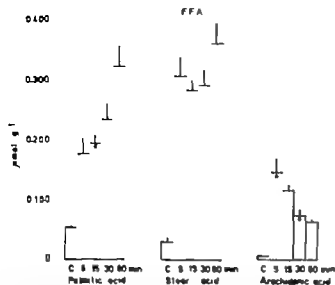


Fig 1 cerebral cortical concentrations of palmitic acid (16:0), stearic acid (18:0) and arachidonic acid (20:4) after 5, 15, 30 and 60 min of isoelectric EEG. The values are given in $\mu\text{mol} \times \text{g}^{-1}$ wet weight and represent means \pm S.E. $P < 0.05$, $P < 0.01$, $P < 0.001$.

values either increased gradually in the period 5–60 min or remained constant following the increase at 5 min. In contrast following a 20-fold rise at 5 min the arachidonic concentration then gradually fell and at 60 min the value was 10 times greater than control.

The results thus demonstrate that severe hypoglycemia leads to an increase in the FFA pool similar to that observed in ischemia. It is of interest that hypoglycemia neither causes a decrease in cerebral oxygen availability nor a reduction of cellular redox systems (for literature see Agardh et al 1978). However like ischemia it leads to extensive deterioration of cerebral energy state with a reduction in ATP and increases in ADP and AMP concentrations. Thus the results corroborate the conclusion that activation of phospholipases occurs when ATP depletion is at hand (Markelonis & Garbus 1975).

Recent results showed that in experiments on renal papillae incubated in vitro the release of prostaglandins was inversely proportional to the glucose concentration of the buffer (Tannenbaum et al 1979). The authors concluded that the formation of prostaglandin was due to release of arachidonic acid from lipid pools in the glucose-deprived tissue.

By analogy we tentatively conclude that glycemia a condition leading to increased arachidonic acid without a reduction in oxygen supply should be accompanied by synthesis of prostaglandins and related substances. Possibly the gradual reduction of arachidonic concentration during the course of hypoglycemic coma reflects such a synthesis.

The skilful technical assistance of Kerstin and Lena Sjöberg is gratefully acknowledged. This work was supported by grants from the Swedish Medical Council (No. 14X 763) from U.S. Public Health (No. RO1 NS07838) and Thonstén and Eklund Foundation.

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Combinations of flow rate in mmHg and gap size in mm used to produce different maximal P_{max} and maximal pressure gradient (G_{max})
 rows as gap size/flow rate

| P_{max} , mmHg | | | | | |
|-------------------------|---------|---------|----------|---------|----------|
| 16 | 22.5 | 30 | 32 | 42 | 45 |
| 0.4/380 | | 0.4/305 | | | |
| | 0.2/260 | | | 0.3/300 | |
| | | | 0.15/240 | | 0.25/440 |

LTS

1 shows the P_{max} and G_{max} for different set flow rate and gap size. P_{max} values up to only 16 mmHg were used. Above this range any P_{max} accompanied by a G_{max} which could not be 1 to any large extent, even with different ratios of flow rate and gap size. For example, a P_{max} of 90 mmHg could only be achieved with rates ranging between 280 and 320 mmHg/mm. However, it was the sum of the present 16 apply a pressure barrier with a constant and widely different G_{max} or vice versa. Even high pressures could only be obtained at higher flows, so there is a correlation between P_{max} and

All points utilized

| | |
|---------|-------------------|
| b_0 | -0.55 |
| b_1 | +0.18; $t=1.92^*$ |
| b_2 | +0.0037 $t=1.32$ |
| F ratio | 8.09** |
| DF | 2/31 |

$P < 0.05$ ** $P < 0.005$ *** $P < 0.001$

Two outliers excluded

| | |
|---------|-----------------------|
| b_0 | -0.27 |
| b_1 | +0.014 $t=5.22^{***}$ |
| b_2 | +0.0004 $t=0.52$ |
| F ratio | 27.5** |
| DF | 2/29 |

The figures showed clearly that in the region of P_{max} and G_{max} tested, P_{max} is the dominant predictor variable for AR, while G_{max} is of insignificant importance—a but not b_2 differs significantly from zero.

DISCUSSION

In previous studies conducted on the same system and run at 22°C axoplasmic flow as partially arrested by a pressure barrier applied to a section of the vagus nerve (Hahnenberger 1978). The present study indicates that in the low pressure range studied (16–45 mmHg) maximal pressure is of crucial importance for the limited transport block observed, the pressure gradient being of little significance. This suggests that the transport that is blocked in the pressure range studied was blocked because structures were collapsed.

These structures might well be the endoplasmic reticulum. In recent years there has been a number of studies in which transported material was localized within the canaliculi of the endoplasmic reticulum. Horse radish peroxidase and acid phosphatase are substances which are transported bidirectionally and are detectable in the endoplasmic reticulum both during anterograde and retrograde transport (Sotelo & Riche 1974; Holtzman et al. 1955). In the experiments by Droz (Droz et al. 1975) on preganglionic axons of the ciliary ganglion

AR found with different combinations of G_{max} are shown in Fig. 2. Group A reveals G_{max} of 16 mmHg and G_{max} of 30 mmHg/mm did not affect axoplasmic flow. However, in with higher P_{max} and G_{max} it is obvious that G_{max} rather than the gradient is decisive for inhibition of transport within the pressure area. apparent when groups with similar P_{max} but not G_{max} are compared, columns C and E rather similar AR values despite quite different G_{max} . The same is true when groups E and F are read. Groups B and C have similar G_{max} but not P_{max} and the AR values are quite different. The data were utilized together calculating a multiple regression according to the formula:

$$AR = a_0 + a_1 P_{\text{max}} + a_2 G_{\text{max}}$$

o calculations were computed: one including both the other excluding the suspected outliers (indicated by brackets in Fig. 1).

o result of the analyses were:

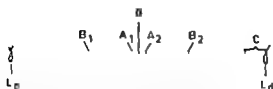


Fig 1 Calculation of the accumulation ratio (AR). A nerve segment between a proximal ligature (L_p) and a distal one (L_d) was subjected to a pressure barrier. The peak of the pressure barrier was situated at the centre of the cap (G). Radioactivity detected in the nerve pieces (2 mm each) immediately adjacent to G (A, A₂) 8–10 mm proximal and distal to G (B₁, B₂) and this accumulated in the 4 mm nerve piece (C) immediately proximal to L_d was used to form AR. For further explanation see text.

ment will be opposed by an existing uphill pressure gradient. If this gradient is large enough, transport might be blocked. On the other hand, if transport occurs within canalicular structures, this will be impeded by "squeezing" the transporting channels until the lumen collapses. This squeezing pressure is defined as the maximal pressure applied to the nerve.

MATERIALS AND METHODS

Animals and anesthesia

Albino rabbits of both sexes weighing between 2.0 and 4.2 kg were used. They were anesthetized by i.v. injection of 7–8 ml/kg of a 2.5% (w/v) aqueous solution of urethane.

Labelling of the motoneurons of the vagus nerve

The Miani procedure (1969) as modified by Fuzell & Sydstrand (1974) was used. The fourth ventricle was exposed and 30–40 μ l of ¹¹I-leucine was applied in 1 μ l drops (1 μ l/ μ l) close to the superior vagus nuclei. 3½ h later 40 to 50 mm of both cervical vagi were removed between two ligatures: the proximal immediately rostral to the ganglion nodosum. After removal the nerves were cleaned from excess connective tissue and then exposed to different experimental conditions. The two nerves of each animal were always subjected to different protocols.

Local compression of the vagus nerve

The method including the superfusion fluid is described in detail in a previous paper (Hahnenberger 1978). Briefly, a constant flow of fluid was driven through an adjustable gap and directed radially on to the whole circumference of a small section of the nerve. The pressure barrier thus applied had a bell-shaped profile, which was characterized by its maximal pressure P_{max} (maximal height of the curve) and the maximal pressure gradient G_{max} (expressed as the steepest slope of the limbs). By adjusting flow and gap size it was possible to obtain different pressures and different gradients constant over an extended period of time. Flow rates and gap sizes providing a particular P_{max} and G_{max} were derived from the calibration curves given in a previous paper (Hahnenberger 1978) rather than determined

for each new set-up. The nerves were superfused at room temperature (22°C) and then cut by a scalpel blade through the gap. The two nerve segments, divided in turn into two mm pieces, which were at least 24 h in ice-cold 10% trichloroacetic acid and then analysed for radioactivity as described.

The whole set-up did not work satisfactorily in experiments. Nerves run when technical errors were apparent were not utilized.

Determination of radioactivity

The TCA soaked nerve pieces were either combusted in a TriCarb Sample Oxidizer (Packard) or deposited on a filter. Radioactivity was determined accordingly and methods. At least 4000 counts were counted.

Presentation of the data

The inhibition of transport was expressed as an accumulation ratio (AR) calculated as follows (see Fig 1): pieces of nerve 2 mm (immediately proximal (A₁, A₂) to the centre of the pressure barrier, the 4 mm immediately proximal to the distal ligature (C) pieces from B₁ to 10 mm on either side of the centre were used for the calculations. A and A₂ represent the immediate vicinity of the pressure peak, B₁ and B₂ represent the part of the nerve not subjected to pressure, the accumulation at the distal ligature. Radioactivity was measured in each piece and the accumulation ratio AR formed as: $AR = (A + A_2 + B_1 + B_2)/C$.

Statistics

The accumulation ratio (AR) may have been affected by both P_{max} (the maximal pressure) and G_{max} (the pressure gradient). Multiple linear regressions were therefore calculated using a HP 9810 A desk calculator program STAT PAC IV-1. Significance was determined with the *t*-test according to the formula of Snedecor (1956).

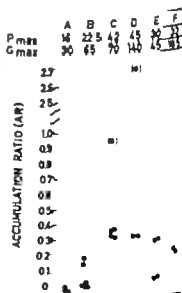


Fig 2 AR with different combinations of P_{max} and G_{max} . The suspected outliers are in brackets.

35 Combinations of flow rate in ml/min and gap size in mm used to produce different maximal
 36 pressures (P_{max}) and maximal pressure gradients (G_{max})
 37 shown as gap size/flow rate

| ml/min | P_{max} mmHg | | | | | |
|--------|----------------|------|---------|----------|---------|----------|
| | 16 | 22.3 | 30 | 33 | 43 | 45 |
| | 0.4/300 | | 0.4/305 | | 0.3/300 | |
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| | | | | | | 0.25/480 |

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 flow rate and gap size. P_{max} values up to only
 45 mmHg were used. Above this range any P_{max}
 is compensated by a G_{max} which could not be
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AR points utilized

| | |
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| F value | 8.09* |
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$P < 0.05$ $P < 0.005$ $P < 0.001$

Two outliers excluded

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$$y = a_0 + a_1 P_{max} + a_2 G_{max}$$

1) calculations were computed, one including
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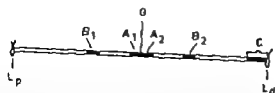


Fig. 1 Calculation of the accumulation ratio (AR). A nerve segment between a proximal ligation (L_p) and a distal one (L_d) was subjected to a pressure barrier. The peak of the pressure barrier was situated at the centre of the cap (G). Radioactivity detected in the nerve pieces (2 mm each) immediately adjacent to G (A_1 , A_2) 8–10 mm proximal and distal to G (B_1 , B_2) and this accumulated in the 4 mm nerve piece (C) immediately proximal to L_d was used to form AR. For further explanation see text.

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The inhibition of transport was expressed in accumulation ratio (AR) calculated as follows (see Fig. 1). Pieces of nerve 2 mm immediately proximal (A_1) and distal (A_2) to the centre of the pressure barrier (G) and immediately proximal to the distal ligation (C) were used for the calculations. A_1 and A_2 represent the immediate vicinity of the pressure peak (B_1 and B_2) and this part of the nerve not subjected to pressure. The accumulation at the distal ligation (C) was re-measured in each piece and the accumulation ratio AR formed as $AR = (A_1 + A_2 - B_1 - B_2) / C$.

Statistics

The accumulation ratio (AR) may have been affected both by P_{max} , the maximal pressure and G_{max} , the maximal pressure gradient. Multiple linear regression was therefore calculated using a HP 9810 A desk calculator program STAT PAC IV-1. Significance was evaluated with the t test according to the formula of Snedecor (1956).

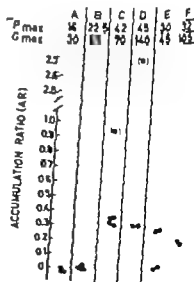


Fig. 2 AR with different combination of P_{max} and G_{max} . The suspected outliers are in bracket.

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in the chicken it was found that fast moving labelled proteins are associated with endoplasmic reticulum. When the nerve was slightly compressed the lumen of the endoplasmic reticulum dilated in front of the compression giving a high grain density. Weiss (1973) has suggested that microtubules might be transporting channels. However it seems reasonable that channels such as endoplasmic reticulum consisting of a considerable lumen surrounded by a relatively thin wall should be easier to collapse under the present experimental conditions than microtubules with a small lumen and a comparatively thick wall. But it cannot be excluded that microtubules collapse too.

Although canalicular transport may exist little is known about the driving force and the mechanism for the intraluminal convection. Probably not only microtubules but also contractile proteins play a role (Bray 1977; Roisen et al 1978).

With increasing P_{max} the inhibition of axoplasmic flow was enhanced. This might be due to more pronounced changes in all axons and/or to an increase in the proportion of affected axons. This problem cannot be solved by the present methods.

An important question is: If the applied pressure can collapse the axon itself. In this case the results would give little insight into intracellular transport systems. It can however reasonably be assumed that endoplasmic reticulum is easier to collapse than the whole axon. In addition collapsing the axon itself (as in a ligature) would probably destroy the integrity of the longitudinally organized axoplasm and its constituents, most likely resulting in irreversible damage to the axon. Axoplasmic transport block by this mechanism should thus be reversible. But it was found that axoplasmic transport did indeed recover when a pressure barrier of 60 mmHg was applied for only four hours (Hahnenberger 1978). It can thus be assumed that the results presented here mainly reflect intracellular processes.

Axoplasmic transport moves intracellular components of widely different sizes, from soluble proteins to organelles such as vesicles, adrenergic granules and mitochondria. Since such organelles have never been described inside the endoplasmic reticulum, canalicular transport is probably confined to small sized material, perhaps only in the soluble fraction. Other transport systems than canalicular convection may thus exist, responsible for transfer of material of greater size. Bárány (1978) found that at 37°C retrogradely transported

adrenergic granules in postganglionic fibres are able to surmount a pressure gradient of 4 mmHg/mm length but not of 30 mmHg/mm. This suggests that under his conditions particulate matter may indeed travel by a sliding process.

The present experiments differ from Bárány by dealing with anterograde transport at 22°C in preganglionic fibres and not confined to particulate matter (adrenergic perikarya). Thus the two results cannot be directly compared. Moreover the data given here are only valid in a range of the rather low pressures tested. It is unfortunately not possible to achieve a pressure barrier with every steep gradient but above a certain pressure. At such a barrier (simulating an eye) the gradient might well be the important parameter. If there exists a pressure step between intraocular pressure and tissue pressure of the nerve (Ernest & Potts 1968). This pressure difference is established within a very small distance, probably less than 0.1 mm. A high maximal pressure could thus be obtained despite a low maximal pressure. Thus it can still not be decided whether a pressure gradient or the pressure step itself can be suspected of causing glaucomatous perikaryopathy.

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A simple and efficient method for studying neurotransmitter release *in vitro* by a radiotracer technique

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WIKBERG J. E. S. & AXELSSON K. L. A simple and efficient method for studying neurotransmitter release *in vitro* by radiotracer technique. *Acta Physiol Scand* 1980, 109: 123-129. Received 21 Sept 1979. ISSN 0001-6772. Department of Pharmacology, Linköping University, Sweden.

A method which allows measurement of neurotransmitter release from isolated preparations has been developed. With this method release of ^3H -acetylcholine from guinea pig ileum and rabbit jejunum could be studied after preincubation of the preparations with ^3H -cholesterol. The release of noradrenaline from guinea pig vas deferens could also be measured after preincubation with ^3H -noradrenaline. This method is based on a device which allows experiments on 4 preparations at the same time including incubation, field stimulation and collection of fractions for counting of radioactivity. With the purpose of obtaining a simple and inexpensive device for stimulating the tissue preparations electrically a physiological stimulator distributor (PSD) was constructed. It is concluded that the described method constitutes an inexpensive simple and quick means of studying neurotransmitter release from isolated tissues.

Release of neurotransmitters from nerves in isolated tissues has frequently been studied with *in vitro* techniques. If the neurotransmitter pools of nerves within the tissue preparation are labelled with a radioactive tracer it is a relatively simple matter to measure the transmitter release. This approach has been used in investigations of adrenergic transmission in a number of tissues (for review see Starke 1977) and also in studies of the release of acetylcholine from guinea pig ileum and rat lacrimal glands (Wikberg 1977, 1979). In these studies a superfusion technique was used in collection of radioactivity released from the preparation. Although convenient in some respects, this has the disadvantages of requiring a rather complex set-up with organ chambers, perfusion pumps and fraction collectors. With such costly and complicated equipment it is often not possible to run multiple experiments simultaneously. In this paper a simple and inexpensive method for studying neurotransmitter release with use of an isotope technique is described. It is demonstrated that release of the cholinergic neurotransmitter can be

measured both from guinea pig ileum and from rabbit jejunum. Measurements can also be made of the release of the adrenergic neurotransmitter from the guinea pig vas deferens. Multiple organs can be studied simultaneously. The handling of the samples has been reduced to a minimum. A preliminary account of this work has been presented previously (Wikberg & Axelsson 1979).

METHODS

Rabbits of either sex or male guinea pigs were killed by a blow on the head and exsanguinated. In some experiments the vasa deferentia were removed and dissected free from attached mesentery. The organs were cut into four 15 mm pieces and mounted on the 4 perspex holders described below. In other experiments the guinea pig ileum or rabbit jejunum was dissected as described previously (Wikberg 1977b) to obtain innervated specimens of the longitudinal muscle layers. These preparations were also mounted on the perspex holders. The holders were then immersed into scintillation vials containing 8 ml of Krebs solution gassed with 95% O_2 and 5% CO_2 and maintained at temperature of 37°C. In order to label the transmitter stores of the adrenergic nerves in the vasa deferentia, these preparations were incubated with 2×10^{-7} M ^3H -noradrenaline

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Journal of Pharmacology, Linköping University, Sweden

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A method which allows measurement of neurotransmitter release from isolated preparations has been developed. With this method release of ^3H -acetylcholine from guinea pig ileum and rabbit jejunum could be studied after preincubation of the preparations with H -cholec. The release of noradrenaline from guinea pig vas deferens could also be measured after preincubation with ^3H -noradrenaline. The method is based on a device which allows experiments on 4 preparations at the same time: loading incubation, field stimulation and collection of fractions for counting of radioactivity. With the purpose of obtaining a simple and inexpensive device for stimulating the tissue preparations electrically, a physiological stimulator distributor (PSD) was constructed. It is concluded that the described method constitutes an inexpensive simple and quick means of studying neurotransmitter release from isolated tissues.

release of neurotransmitters from nerves in isolated tissues has frequently been studied with open techniques. If the neurotransmitter pools of nerves within the tissue preparation are labelled with a radioactive tracer it is a relatively simple matter to measure the transmitter release. This approach has been used in investigations of adrenergic neurotransmission in a number of tissues (for review see Starke 1977) and also in studies of the release of acetylcholine from guinea pig ileum and bovine lacrimal glands (Wikberg 1977a, 1979). In these studies a superfusion technique was used in collection of radioactivity released from the preparation. Although convenient in some respects, the disadvantages of requiring a rather complex set-up with organ chambers, perfusion pumps and fraction collectors. With such costly and complicated equipment it is often not possible to run multiple experiments simultaneously. In this paper a simple and inexpensive method for studying neurotransmitter release with use of an isotope technique is described. It is demonstrated that release of the cholinergic neurotransmitter can be

measured both from guinea pig ileum and from rabbit jejunum. Measurements can also be made of the release of the adrenergic neurotransmitter from the guinea pig vas deferens. Multiple organs can be studied simultaneously. The handling of the samples has been reduced to a minimum. A preliminary account of this work has been presented previously (Wikberg & Axelsson 1979).

METHODS

Rabbits of either sex or male guinea pigs were killed by a blow on the head and exsanguinated. In some experiments the vas deferens were removed and dissected free from attached mesentery. The organs were cut into four 15 mm pieces and mounted on the 4 peristaltic holders described below. In other experiments the guinea pig ileum or rabbit jejunum was dissected as described previously (Wikberg 1977b) to obtain innervated specimens of the longitudinal muscle layers. These preparations were also mounted on the peristaltic holders. The holders were then incubated in a stimulation vial containing 8 ml of Krebs solution gassed with 95% O_2 and 5% CO_2 and maintained at a temperature of 37°C. In order to label the transmitter stores of the adrenergic nerves in the vas deferens, these preparations were incubated with 2×10^{-7} M [^3H]-noradrenaline

A simple and efficient method for studying neurotransmitter release *in vitro* by a radiotracer technique

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A method which allows measurement of neurotransmitter release from isolated preparations has been developed. With this method release of ^3H -acetylcholine from guinea pig ileum and rabbit jejunum could be studied after preincubation of the preparations with ^3H -cholesterol. The release of noradrenaline from guinea pig vas deferens could also be measured after preincubation with ^3H -noradrenaline. The method is based on a device which allows experiments on 4 preparations at the same time including incubation, field stimulation and collection of fractions for counting of radioactivity. With the purpose of obtaining a simple and inexpensive device for stimulating the tissue preparations electrically, a physiological stimulator distributor (PSD) was constructed. It is concluded that the described method constitutes an inexpensive simple and quick means of studying neurotransmitter release from isolated tissues.

Release of neurotransmitters from nerves in isolated tissues has frequently been studied with open techniques. If the neurotransmitter pools of nerves within the tissue preparation are labelled with a radioactive tracer it is a relatively simple matter to measure the transmitter release. This approach has been used in investigations of adrenergic neurotransmission in a number of tissues (for references, see Starke 1977) and also in studies of the release of acetylcholine from guinea pig ileum and rabbit lacrimal glands (Wikberg 1977, 1979). In the studies, superfusion technique was used in collection of radioactivity released from the preparation. Although convenient in some respects, it has the disadvantages of requiring a rather complex set-up with organ chambers, perfusion pumps and fraction collectors. With such costly and complicated equipment it is often not possible to run multiple experiments simultaneously. In this paper a simple and inexpensive method for studying neurotransmitter release with use of an isotope technique is described. It is demonstrated that release of the cholinergic neurotransmitter can be

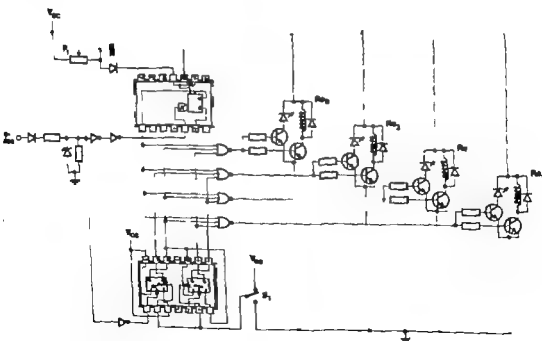
measured both from guinea pig ileum and from rabbit jejunum. Measurements can also be made of the release of the adrenergic neurotransmitter from the guinea pig vas deferens. Multiple organs can be studied simultaneously. The handling of the samples has been reduced to a minimum. A preliminary account of this work has been presented previously (Wikberg & Axelsson 1979).

METHODS

Rabbits of either sex or male guinea pigs were killed by blow on the head and exsanguinated. In some experiments the vasa deferentia were removed and dissected free from attached mesentery. The organs were cut into four 15 mm pieces and mounted on the 4 perspex holders described below. In other experiments the guinea pig ileum or rabbit jejunum was dissected as described previously (Wikberg 1977b) to obtain isolated specimens of the longitudinal muscle layers. These preparations were also mounted on the perspex holders. The holders were then immersed into a scintillation vial containing 3 ml of Krebs solution gassed with 95% O_2 and 5% CO_2 and maintained at temperatures of 37°C. In order to label the transmitter stores of the adrenergic nerves in the vasa deferentia, these preparations were incubated with $2 \cdot 10^{-6}$ M ^3H -noradrenaline



Fig. 2 Photograph of the experimental set up. Incubation is performed by immersing the perspex holders into acclimation vials contained in thermostated water bath. The vials are mounted at their bottoms to stainless steel holders. The incubation medium is delivered by three way automatic syringe



3 A schematic diagram of the physiological stimulator distributor. V_{cc} = supply voltage (+5V). P = transformer. R_1 = relay 0-3. Prepulse indicates trigger output from the Grass stimulator. S_1 = resect switch.

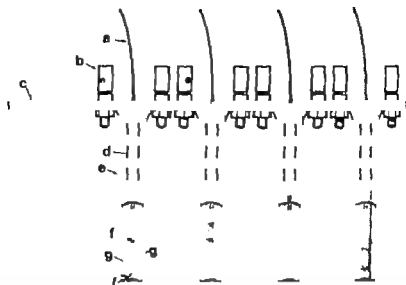


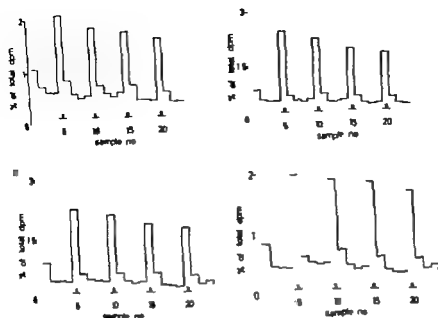
Fig. 1. Schematic drawing of the perspex holders. (a) Polyethylene tubing for oxygen inlet. (b) Electrical terminal for connection to the PSD. (c) Perspex board. (d) Perspex tube. (e) Insulated platinum wire. (f) Insulated bars to which the preparation is tied. (g) Platinum electrodes.

(New England Nuclear 60 Ci/mol) and $\times 10^{-6}$ M ascorbic acid for 1 h. To label the transmitter stores of the cholinergic nerves in guinea pig ileum and rabbit jejunum these preparations were incubated with 1×10^{-6} M H-choline (Amersham 13.2 Ci/mol) for 1 h. After incubation the vaso deferentia preparations were washed with Krebs solution containing 2×10^{-6} M desmethylnoraprine and 1×10^{-6} M normethanephrine. The intestinal preparations were washed with Krebs solution containing 1×10^{-6} M heptabolinum. Electrical stimulation was performed with a Grass SD9 stimulator and a physiological stimulator distributor (PSD) described below. The stimulating current was adjusted to 150 mA. Higher currents than 300 mA resulted in irreversible damages to the tissues. At the end of the experiments the preparations were dissolved in 0.5 ml solubene (Packard) and the radioactivity was counted in a toluene based scintillator (counting efficiency 40–45%) in a Packard Tri Carb 3375 liquid scintillation spectrometer. The radioactivity in the washing medium was counted after the addition of 10 ml of Instagel (Packard) with a counting efficiency of 18–20%. The counting efficiencies were determined by use of an internal standard.

Perspex holders. The organs were mounted on the 4 perspex holders shown in the schematic drawing in Fig. 1. These holders are constructed from four 75-mm long perspex tubes with an inner diameter of 6 mm. Two 15-mm long platinum electrodes are attached at the distal end. The preparation is mounted in between these electrodes by tying them to the (insulated) bars situated above and below the electrodes. The 4 holders are mounted at their proximal ends to a perspex board at which electrical terminals for cables from the PSD can be connected. The platinum electrodes are electrically connected to these terminals by insulated platinum wires. The incubation media can be oxygenated through polyethylene tubings mounted on each holder.

For incubating and washing of the preparation holders are immersed (manually) in scintillation vials containing the appropriate medium. For this purpose they are mounted onto a stainless steel holder contained in a thermostated water bath (Fig. 1). These holders were originally used in an agitating machine, but were chased from Grants Instrument (England) for collection of radioactivity released from the preparation. The specimens are washed by manually dipping the perspex holders into new vials. The washing periods used in the present experiments were 5 min when the guinea pig deferens was studied and 5 min in the experiments on the testicular preparations.

Physiological stimulator distributor (PSD). The effect of the SD9 stimulator was too low to allow the preparations to be stimulated at the same time. We therefore constructed a simple and inexpensive device to solve this problem. Thus, in the PSD the signal from the stimulator is multiplexed to 4 output ports by appropriately timed reed relays. The timing of the relays is achieved by the TTL circuitry shown in Fig. 3. The prepulse of the SD9 is fed to a divide-by-four counter, constructed from two JK flip-flop circuits and a monostable multivibrator (see TTL data book). By decoding the output of these circuits in 4 three-input NAND gates one of the relays can be switched on before the SD9 stimulus pulse is emitted. After the emission of the pulse of the organ the relay is turned off when the multivibrator resets. On the next prepulse this scheme is repeated, now the next relay is activated. This process continues as long as the SD9 is emitting pulses. The device also contains 4 light emitting diodes which are active when the reed relays are turned on, which allows a convenient check that the device is functioning properly (a copy of the PC-board and parts list of the PSD are obtained on request).



6 Spontaneous and stimulation-evoked release of tritium from four guinea pig vas deferens preparations pretreated with ^3H -noradrenaline. S = stimulation with 400 volleys at 4 Hz.

in an identical manner as above. Electrical field stimulation at 1 Hz for 5 min with 1 ms volleys led to an increase in the release of tritium (Fig. 5). However, the stimulation-evoked release was less pronounced than that in guinea pig Deans. To test if the preparation responded to pharmacological agents the effect of noradrenaline was examined, as it has been previously shown that this drug blocks the release of acetylcholine from the rabbit ileal intestine as estimated by bioassay (Vizi & Old 1971). Noradrenaline (1×10^{-6} M) completely blocked the stimulation-evoked release of tritium (Fig. 5b).

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Incubation of the preparations with ^3H -noradrenaline they were washed at 2-min intervals for 60 s. Electrical field stimulation was then performed 10-min intervals with 400 volleys at 4 Hz with 1.5 pulses. The fractions collected during the first stimulation period were discarded, as the release during this period was much larger than during the following ones. After this initial stimulation, fractions were collected for analysis. The electrical stimulation gave rise to a large and consistent increase in the release of tritium. The release was

comparable in magnitude in all 4 preparations studied simultaneously (Fig. 6). To test that the preparations responded pharmacologically the effect of 8×10^{-6} M phentolamine was examined. It is well known that this drug potentiates transmitter release on nerve stimulation (for references see Starke 1977). The α -blocking agent induced an approximately 3-fold potentiation of the tritium release on field stimulation (data not given). On no occasion was any spurious release of tritium observed.

DISCUSSION

In this paper we have described a method by which the release of noradrenaline or acetylcholine from isolated tissues can be studied as release of labelled transmitter. The cost of the equipment was only a fraction of that of conventional experimental set up with the superfusion technique. Furthermore the present method allows multiple preparations to be studied simultaneously. The handling of the samples was also reduced to a minimum, as incubation and counting of radioactivity are performed in the same vessels. Since a larger amount of experimental data can thus be obtained in a shorter time

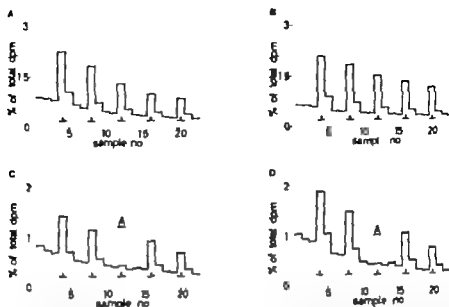


Fig 4 Spontaneous and stimulation evoked release of tritium from four guinea pig ileum preparations preincubated with H-choline. ▲ = stimulation with 150 volleys at 0.5 Hz. (A-B) Control (C-D) Addition of 2×10^{-4} M adenosine (A) as indicated

Drugs The following were used. Adenosine (Boehringer Mannheim) hemicholinium bromide (EGA Chemie) and noradrenaline bitartrate (Sigma)

RESULTS

Guinea pig ileum

After incubation of the guinea pig ileum preparations with ^3H -choline they were washed 6 times at 5-min intervals for 30 min by incubation in scintillation vials. This initial washing was undertaken with the purpose of washing out extracellularly located metabolites (Wikberg 1977a). After this time fractions were collected for 5 min each for analysis. Electrical field stimulation with 150 pulses at 0.5 Hz and a duration of 1 ms induced a consistent increase in the release of tritium (Fig 4). The tritium release from the 4 preparations studied in the same experiment was comparable in magnitude and on no occasion was any spurious release of tritium observed. In order to check that response to pharmacological agents occurred with use of the present method two of the preparations were treated with 2×10^{-4} M adenosine since this drug has been reported to inhibit the release of acetylcholine from guinea pig ileum as studied by bioassay (Vizi & Knoll 1976) or by gas chromatography mass spectrometry (Gustafsson et al 1979). The stimulation-evoked release of tritium was completely abolished by the addition of adenosine (Fig 4c-d).

Rabbit jejunum

After incubation of the rabbit jejunum preparations with H-choline they were washed 6 times at 5 min intervals for 30 min as described above for guinea pig ileum. Fractions were then collected for 5 min

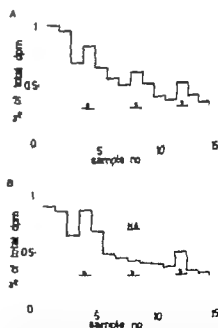


Fig 5 Spontaneous and stimulation evoked release of tritium from two rabbit jejunum preparations preincubated with H-choline. S = stimulation with 300 volleys at 1 Hz. (A) Control (B) Treatment with 1×10^{-4} M noradrenaline (NA) as indicated

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as compared with the conventional techniques the cost of the experiments is reduced. We have chosen to construct a device which allows 4 preparations to be studied simultaneously and we have found that it functions satisfactorily. The equipment is very simple to handle. However, should its capacity be insufficient, there is nothing to prevent a similar apparatus being set up for the study of more preparations, for example 8. The only disadvantage that could be present with the method is that measurement of mechanical tension of the muscle preparation cannot be performed. Due to the complexity of a device which also allowed tension studies we did not implement this facility.

We also developed a device to allow simultaneous stimulation of the preparations. Naturally several Grass stimulators could be used to achieve the same results, but such equipment is rather expensive and it was our aim to develop an inexpensive method. The physiological stimulator distributor cost less than 1/10 of one physiological stimulator.

In our experiments guinea pig ileum released an increased amount of tritium on electrical stimulation. It has been shown that in the presence of eserine the stimulation-evoked release of tritium is almost exclusively attributable to ^3H -acetylcholine (Wikberg 1977a). We did not use eserine and the released acetylcholine was therefore probably hydrolyzed to ^3H -choline. In order to be assured that all of the released tritium was collected, hemicholinium was added to the incubation medium to block neural reuptake of ^3H -choline. The stimulation-evoked release of tritium would thus seem valid as a measure of stimulation-evoked release of ^3H -acetylcholine. Theoretically there may be some problems involved in the use of the radiotracer technique, as it may not be certain that the stimulation-evoked release of tritium correlates to the release of endogenous acetylcholine. However, it has recently been shown that the release of ^3H -acetylcholine observed with the radiotracer method correlated well to the release of endogenous acetylcholine measured by gas chromatographic mass spectrometry (Wikberg et al. in preparation). Regarding the present technique it should be pointed out that some difficulties might be encountered in studies of the spontaneous release of tritium. For instance, it could be rather difficult to predict the amount of ^3H -acetylcholine released into the washing medium. The reason for this is that other metabolites such as ^3H -phosphorylcholine and ^3H -

choline may be released from radioactive preparations which are probably partly contained in the muscle of the preparations (Szerb 1975, Wikberg 1977a). Thus, in studies of the spontaneous release of ^3H -acetylcholine the metabolites should be rated by some chemical method.

Our experiments have also shown that the radiotracer method for measuring the release of acetylcholine is applicable to the rabbit jejunum. Results obtained with noradrenaline-induced stimulation of adrenergic receptors caused a stimulation of acetylcholine release from the cholinergic neurons of this preparation. This is compatible with previous results obtained in studies on mechanical tension in rabbit jejunum which indicated that α -receptor stimulation elicited acetylcholine release (Wikberg 1977b).

The method described was also found suitable for studying the release of ^3H -noradrenaline from vas deferens. The results of these experiments compare favourably to those obtained by several different authors with use of the superfusion technique (Hedqvist 1974, Wikberg & Aaden 1978). It is concluded therefore that the present approach can be used as an equivalent or safe alternative to previous methods.

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Effect of sensitization and non antibody IgE on 48/80 induced histamine release from isolated mast cells

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SYDBOM A., KARLSSON T & UVNÄS B. Effect of sensitization and non-antibody IgE on 48/80 induced histamine release from isolated rat mast cells. *Acta Physiol Scand* 1980; 109: 131-136. Received 27 Sept. 1979. ISSN 0001-6772. Department of Pharmacology, Karolinska Institute, Stockholm, Sweden and Department of Medical and Physiological Chemistry, Biomedical Centre, Uppsala, Sweden.

Histamine release from isolated rat mast cells from non-immunized and immunized Hooded Lister rats was induced by compound 48/80. The histamine release was decreased with lower maximum at the optimal concentration of 48/80 when using cells from immunized rats compared to non-immunized control rats. The stimulation of IgE antibody production after sensitization using *B. pertussis* as an adjuvant was also accompanied by an elevation of total serum IgE. The 48/80 induced histamine release from Sprague Dawley mast cell was not inhibited by immunization. Non-antibody IgE showed non-competitive inhibition of 48/80 induced histamine release when myeloma IgE was incubated with mast cells from both Hooded Lister and Sprague Dawley rats. The results indicate the existence of different receptors for IgE and 48/80.

Key words: *B. pertussis* vaccine, Hooded Lister rats, immunization, Sprague Dawley rats

aphylactic histamine release from mast cells is initiated by immunoglobulin (IgE) (Ishizaka & Ishizaka 1966, Johansson & Benmich 1967). The IgE molecule is bound to specific receptors on the mast cell membranes (Conrad et al 1975). The bridging of two IgE molecules on the mast cell surface by antigen starts the sequence of events that finally leads to the secretion of histamine and other mediators (Levine 1965, Strömberg et al 1975, Segal et al 1977).

Compound 48/80, a non-immunological and non-toxic releaser of histamine (Johnson & Moran 1969, Bloom & Chakravarty 1970) has been used for many years to study the mechanisms of histamine release from isolated rat mast cells. The histamine release induced by 48/80 has many features in common with that of antigen both morphologically and biochemically (Johnson & Moran 1969, Bloom & Chakravarty 1970). However, there are some differences for example in the influence by e.g. Ca^{2+} (Orrenius & Mörner 1972, Uvnäs & Thon 1961), ^{45}P (Becker & Austen 1966, Rasmussen & Cochrane 1971, Taylor & Sheldon 1974) or cyclic nucleotides

(Johnson et al 1974, Kalmer & Austen 1974, Sullivan et al 1975, Fredholm et al 1976, Norn et al 1977) which have raised the question whether there are initially different points of attack for antigen and 48/80 (Uvnäs 1976).

The present investigation was undertaken to study the possible different points of attack for the histamine release mediated by IgE or 48/80 and in particular if changes in the amount of cell bound IgE modifies 48/80 induced histamine release.

MATERIALS AND METHODS

Inbred Hooded Lister rats originating from rats purchased from Møllegaard Avlslaboratorium ApS, L. Skensved, Denmark, and Sprague Dawley rats from Anticimex, Stockholm, Sweden, were used. *Pertussis* vaccine (lot no. 120274) was purchased from Statens Serum Institut, Copenhagen, Denmark (see Sydbom & Karlsson 1979). Egg albumin from Difco Laboratories, Detroit, Michigan, USA and human serum albumin (free of preservatives) from Kabi AB, Stockholm, Sweden, Ficoll from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and compound 48/80 from AB Leo, Helsingborg, Sweden. The IgE was originating from IgE (IR2) secreting tumours in

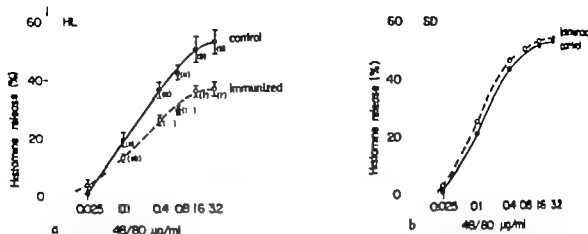


Fig. 1 Effect of immunization on 48/80 induced histamine release from mast cells. Isolated mast cells from non-immunized (●—●) or immunized (○—○) rats were incubated with compound 48/80 (0.025 to 3.2 $\mu\text{g/ml}$) at 37°C for 10 min. (a) Mast cells from Hooded Lister rats. Mean \pm S.E. 1. Number of expts. are shown in brackets. With 48/80 concentrations above 0.4 $\mu\text{g/ml}$ there is a significant difference between control and immunized mast cells ($p < 0.01$). Spontaneous histamine release was $8 \pm 0.8\%$ for non-immunized and $9.3 \pm 1.0\%$ for immunized rats. (b) Mast cells from Sprague Dawley rats. Mean of 2 expts. Spontaneous histamine release was 4.9% for non-immunized and 6.1% for immunized rats.

LOU/c rats reported by Bazin et al. (1973). The monoclonal IgE protein was isolated and purified as described by Bazin et al. (1974). All other chemicals were obtained from the usual commercial sources.

Immunization of rats

Male Sprague Dawley rats (180–200 g) or Hooded Lister rats (220–370 g) were injected s.c. with $\times 0.5$ ml of B pertussis vaccine together with egg albumin (70 $\mu\text{g/ml}$). The animals were killed for isolation of mast cells between day 13 and day 77 after immunization.

Isolation of mast cells

Mast cells from the pleural cavities of Hooded Lister or Sprague Dawley rats were isolated on Ficoll according to the method of Thon & Uvnäs (1967), as used earlier (Sydham & Uvnäs 1976).

Incubation procedure

Standard incubation. The isolated mast cells were suspended in 2 ml incubation medium: 137 mM NaCl, 7 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 containing 10% (v/v) 0.67 M Sørensen's phosphate buffer pH 6.7 and human serum albumin 0.5 mg/ml. The mast cell concentration was usually around 5000 cells/ml. The mast cell suspension was preincubated at 37°C for 5 min and then 100 μl of a compound 48/80 solution was added to the cell suspension and incubated for another 10 min. The reaction was stopped by immersing the tubes in ice-water and centrifuging (340 $\times g$) at 4°C for 10 min. The supernatants were decanted off into new tubes and 0.2 ml 0.1 M HCl was added to the cell residues and the tubes put in boiling water bath for 5 min to extract all histamine. 1.8 ml of buffer was added to the cell tubes to readjust the volume. Histamine was determined according to the fluorescence technique described by Shore et al. (1959), omitting the extraction procedure (Bergendorff & Uvnäs 1977). His-

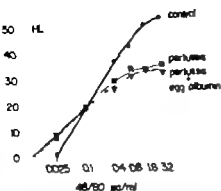
tamine release was expressed as % of total histamine in cell and supernatant. Samples were run in duplicate; the spontaneous histamine release has been subtracted from the values presented.

Competition between IgE and 48/80. Immunophorb (IR2) from a concentrated stock solution (40 mg/ml) incubated with normal Hooded Lister mast cells (1.8×10^6 cells) in 100 μl at 37°C for 30 min during or without shaking. 4 tubes were run parallelly with 0, 0.1, 0.1 and 4 mg IgE respectively. After the incubation 5.4 ml buffer was added and the cell suspension distributed among 10 new tubes each and centrifuged 10 min. The supernatant were discarded. The mast cells were preincubated with 1 ml buffer at 37°C for 5 min with the addition of 100 μl of 48/80 in 4 different concentrations (final conc. 1.6, 0.4, 0.1, 0.025 $\mu\text{g/ml}$) for 10 min. The reactions were stopped and the samples analysed as described above.

RESULTS

48/80 induced histamine release from mast cells from non-immunized and immunized Hooded Lister and Sprague Dawley rats

Isolated mast cells from non-immunized Hooded Lister rats were incubated in increasing concentrations of compound 48/80 at 37°C for 10 min and histamine release was determined (Fig. 1a). There was a maximal histamine release of about 60% using mast cells from non-immunized rats. The dose-response curve was significantly shifted downwards when using mast cells from immunized



Dose-response curve of 48/80 on histamine release. Hooded Lister rat mast cells. Isolated mast cells non-immunized (control) rats (●—●), rats immunized with egg albumin and B pertussis (▼—▼) or B pertussis alone (▲—▲) were incubated with 48/80 (0.0025 to 3.2 µg/ml) at 37°C for 10 min. Mean \pm S.E.

and the maximum was decreased to about 36% difference in the responsiveness of mast cell non-immunized and immunized Hooded rats could however not be shown with mast cells from Sprague Dawley rats (Fig. 1b). The dose at maximum histamine release can be due overall increase in the IgE production. Therefore group of Hooded Lister rats were immunized with B pertussis and egg albumin as before and group of rats were injected with B pertussis as a control. B pertussis is known to be a factor of IgE production in rats (Mota 1958

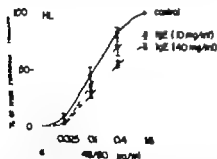
Tada et al. 1972). There was a decrease in histamine release after 48/80 challenge of mast cells from both these immunized groups, as compared to non-immunized control rats but no difference between rats immunized with B pertussis + egg albumin or B pertussis alone (Fig. 2).

Inhibition of 48/80 induced release from mast cells by non-antibody IgE

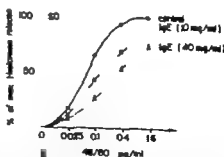
Isolated mast cells were incubated in 100 µl with purified non-antibody myeloma IgE (IR2) in 3 different concentrations 1, 10 and 40 mg/ml at 37°C for 30 min. After dilution and washing the mast cells were incubated with 48/80 0.0025 to 1.6 µg/ml. Log dose of 48/80 was plotted against % of maximal histamine release. High concentrations of non-antibody IgE inhibited the 48/80 induced histamine release from normal Hooded Lister rat mast cells (Fig. 3). There was no effect of the lowest dose of IgE (1 mg/ml) on 48/80 induced histamine release but the dose-response curve was shifted to the right and the maximum was decreased to 80% when the mast cells had been incubated with 10 mg/ml of IgE and to about 70% when 40 mg/ml of IgE was used (Fig. 3). The picture was very much the same when mast cells from Sprague Dawley rats were used (Fig. 3b).

DISCUSSION

The original finding of this work was that an overall stimulation of IgE production in Hooded Lister rats by immunization with egg albumin and B per



3a Effect of IgE on 48/80 induced histamine release. Isolated mast cells (4×10^6 cells) were incubated in 100 µl with purified myeloma IgE in two different concentrations, 10 mg/ml (▲—▲) or 40 mg/ml (△—△) and a control (●—●) at 37°C for 10 min. After washing and dilution the mast cells were incubated with 48/80 in 4 different concentrations at 37°C for 10 min. The histamine release is expressed as percent of maximal release with 1.6 µg/ml 48/80 but rats. (a) Histamine release from Hooded Lister rat mast cells. Mean \pm S.E. from 4 expts. (b) Histamine release from Sprague Dawley rat mast cells. Mean \pm S.E. from 2 expts.



tussis or B. pertussis alone inhibited 48/80 induced histamine release from isolated rat mast cells (Fig. 1a). The maximal histamine release response was decreased about 30% by immunization. However we could not register any difference in histamine release between non-immunized or immunized Sprague Dawley rats. Sprague Dawley rats have very low basic levels of IgE (0–10 ng/ml) compared to Hooded Lister rats (about 600 ng/ml) (Karlsson et al 1979). There are only small variations in the total IgE level after immunization of Sprague Dawley rats (5–10 ng/ml Bennich et al 1978) but the level of total IgE in Hooded Lister rats is increased to about 2000 ng/ml (Sydbom & Karlsson 1979). This very pronounced difference in IgE between the two rat strains could perhaps explain why the histamine release induced by 48/80 could be decreased by immunization of Hooded Lister rats but not Sprague Dawley rats. To investigate whether this inhibition was due to IgE we incubated the mast cells with purified non-antibody IgE from IgE secreting myeloma cells. The results showed that there was a non-competitive inhibition of 48/80 induced histamine release with increasing IgE both when mast cells from Hooded Lister rats and Sprague Dawley rats were used. However if we calculate the IgE in the incubation medium there is a great excess of IgE. The molecular weight of rat IgE is about 190 000 (Bennich et al 1978) and the number of receptors on a mast cell surface around 300 000 (Conrad et al 1975). In our experiments we used 4 mg of myeloma IgE to 380 000 mast cells which will give 1.7×10^6 molecules of IgE per receptor. With this excess all receptors ought to be occupied and it is rather surprising that there could be a differentiation between the inhibitory effect of the two doses of IgE: 10 and 40 mg/ml.

If the amount of IgE molecules attached to the mast cell receptors is a reflection of the serum level of IgE, the immunized Hooded Lister mast cell might be covered by IgE and there would probably be a steric hindrance for 48/80 to react with its receptor and the dose response curves indicated a non-competitive inhibition (Fig. 3). The serum level of IgE in immunized Sprague Dawley rats is low and the concentration of IgE molecules on the mast cell surface consequently low. This could explain why immunization of Sprague Dawley rats does not inhibit 48/80 induced histamine release (Fig. 1b). The number of IgE molecules on human basophils correlate well with the serum IgE level (Conroy et

al 1977). Sobotka & coworkers (1978) keep the number of basophil receptors also constant the serum IgE levels. However mast cells from non-immunized Sprague Dawley rats have free IgE receptors than Hooded Lister rats; there is no difference in the total uptake of IgE between mast cells from Hooded Lister and Sprague Dawley rats (Karlsson 1978) indicating that total number of IgE receptors are the same in two rat strains.

Hmo et al (1977) have reported evidence of specific binding of 48/80 to mast cell surface would indicate the existence of a 48/80 receptor. However Morrison et al (1975) have made preliminary experiments that would suggest that IgE to act on the same receptor. This was drawn out of desensitization studies: mast cells from immunized rats and desensitized antigen did not respond to 48/80. In view of this this could perhaps also be explained by hindrance by IgE. In conclusion our findings that a stimulation of IgE production in Hooded Lister rats inhibits 48/80 induced histamine release is a fact that non-antibody IgE non-competitively inhibits 48/80 induced histamine release indicating existence of different receptors for IgE and different points of initial attacks for the inhibition of secretion.

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β - γ -methylene-ATP and adenosine inhibit cholinergic non-adrenergic transmission at urinary bladder

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DAHLÉN S.-E. & HEDQVIST P. ATP, β - γ -methylene-ATP and adenosine inhibit non-cholinergic non-adrenergic transmission in rat urinary bladder. *Acta Physiol Scand* 1980, 109: 137-142. Received 1 Oct. 1979. ISSN 0001-6772. Department of Physiology, Karolinska Institute, Stockholm, Sweden.

ATP and adenosine caused dose-dependent and reversible inhibition of the atropine resistant contraction response to transverse nerve stimulation in the rat urinary bladder. Both purines also inhibited contraction responses to acetylcholine and direct muscle stimulation, indicating postjunctional effects on the transmission. It seems as ATP per se inhibits the excitatory transmission, because the stable ATP-analogue β - γ -methylene-ATP was inhibitory as well, and because exogenous adenosine decreased the inhibition by adenosine but not that by ATP or β - γ -methylene-ATP. Blockade of purine inactivation enhanced the inhibitory action of ATP and adenosine, and by itself inhibited the transmission. These results are consistent with the possibility that endogenous purines may modulate non-cholinergic non-adrenergic excitatory transmission in the rat urinary bladder.

Key words: ATP analogue, β - γ -methylene-ATP, non-cholinergic non-adrenergic transmission, rat urinary bladder, blockade of purine inactivation, transverse nerve stimulation.

is considerable morphological evidence that innervation urinary bladder is cholinergically ried (e.g. El-Badawi & Schock 1966). Yet, it is well established that atropine fails to block -induced contractions in the urinary bladder (Langley & Anderson 1896). This phenomenon commonly been explained in terms of an atropine-resistant transmission with junctional receptors that are inaccessible to atropine (Dale & Glad 1930; Carpenter 1977). However, it has also suggested that part of the excitatory transmission is non-cholinergic (Henderson & Roepke 1971) and, more specifically, "purinergic" (Dunlop 1971; Burnstock et al. 1972, 1978). ATP undoubtedly contracts the urinary bladder of most mammals (e.g. Buchthal & Rablson 1944) and ATP is released by intramural nerve stimulation in the guinea pig urinary bladder (Burnstock et al. 1978). Still, the hypothesis of purinergic transmission has been challenged mainly on the basis that ATP-contractions in many respects

imperfectly mimic the response to nerve stimulation (Ambache & Zar 1970; Ambache et al. 1977; Ambache 1978).

Purines and in particular adenosine have been suggested as modulators of adrenergic and cholinergic transmission (Hedqvist & Fredholm 1976; Sawynok & Jhaumandas 1976; Vizi & Knoll 1976). We report that ATP and its degradation product adenosine and the stable ATP-analogue β - γ -methylene-ATP (β - γ -M-ATP) inhibited nerve-induced contractions in the isolated rat urinary bladder which raises the possibility that endogenous purines may modulate non-cholinergic non-adrenergic transmission as well.

A preliminary account of part of the results was presented at the XVI Scandinavian Congress of Physiology and Pharmacology 1979 (Dahlén 1979).

MATERIAL AND METHODS

Female Sprague-Dawley rats (250 g), were killed by blow on the head and bled. The urinary bladder was

isolated and two mucosa-free strips prepared from the detrusor part. Unstretched the strips were approximately 5×15 mm with a thickness of 1–3 mm. The preparation was suspended in a 5 ml organ bath containing Krebs-Henseleit's solution (composition (mM): NaCl 143, KCl 5.0, CaCl_2 2.5, MgCl_2 1.0, NaHCO_3 11.9, glucose 5.5). The solution was kept at 37°C and gassed with 5% CO_2 in O_2 . Washout was performed by changing the whole bath volume. Unless otherwise stated, biphasic pulses of 0.5 ms duration and of supramaximal voltage were delivered by a Grass S4 stimulator through platinum wire electrodes along the wall of the organ bath. Contractions were recorded on a Grass Model 7 Polygraph via an isometric Grass FT 03C force displacement transducer (resting tension 5–10 mN). In some experiments, mechanical activity was registered isotonicity (load 0.5 g) with a Harvard heart/smooth-muscle transducer on a Honeywell ink writer. As regards the inhibitory action of ATP and adenosine, both recording methods gave the same result.

Drugs (reagent grade): acetylcholine chloride (ACh, Sigma), atropine sulfate (Sigma), tetrodotoxin (Sigma), hexamethonium bromide (May & Baker Ltd), adenosine 5'-triphosphate disodium salt (ATP, Sigma), adenosine (Sigma), β - γ -methylene adenosine 5'-triphosphate sodium salt (β - γ -M-ATP, Sigma), dipyrindamole (provided by Boehringer Ingelheim AB), guanethidine bisulfate (provided by Hässle-Ciba-Geigy AB), adenosine deaminase (ADA, Sigma, type II crude powder, Act IU $1 \mu\text{mol}$ adenosine/min, pH=7.5, $T=25^\circ\text{C}$), erythro-9-(2-hydroxy-3-nonyl)-adenine (=BHNA, provided by Burroughs-Wellcome). Since atropine had little and guanethidine no effect on the contraction response to transmural nerve stimulation, these drugs were not routinely added to the bath fluid.

The effect of drugs on the response to transmural nerve stimulation was calculated from the ratio between the average height of 5 contractions immediately preceding drug addition and that of 5 contractions after a stable drug effect was obtained. Statistical hypotheses were tested by Student's *t* test for paired and unpaired variates.

RESULTS

Strips of rat detrusor muscle consistently responded to transmural stimulation (1–200 pulses, 1–40 Hz) with a prompt contraction. This contraction was abolished by tetrodotoxin ($0.31 \mu\text{M}$) but left unaffected by guanethidine ($1\text{--}5 \mu\text{M}$) or hexamethonium ($3\text{--}30 \mu\text{M}$). After atropine ($0.1\text{--}1.0 \mu\text{M}$) the contraction caused by ACh ($0.01\text{--}100 \mu\text{M}$) was completely blocked, while the contraction response to transmural stimulation (10 pulses, 1–5 Hz) was at most reduced by 30% (mean \pm S.E. = $13.8 \pm 3.0\%$, $n=11$, $P<0.001$). Thus the contraction obtained by transmural stimulation of rat urinary bladder is nerve-mediated, resistant to adrenergic and ganglionic blockade and only moderately reduced by blockade of muscarinic receptors.

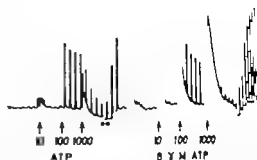


Fig. 1. Dual effect of ATP and β - γ -M ATP on rat urinary bladder direct contraction but inhibitory responses to transmural nerve stimulation (5 Hz, 10 pulses) at 1 min intervals. Drugs added at arrows and dots.

This is in agreement with previous findings in rat species including the rat (e.g. Ambache & 1970).

ATP ($1\text{--}15000 \mu\text{M}$) initially caused a rapid and dose-dependent contraction of the rat detrusor strip, but there was always a marked tachyphylaxis to repetitive administration of one and the same dose of ATP. The contraction response to subsequent transmural nerve stimulation (10 pulses, 1 at 1 min intervals) was however never enhanced but rather dose-dependently depressed (Fig. 1). The inhibitory effect which shows tachyphylaxis was manifest within 30 s and reached its maximum within 2–3 min and remained so until the preparation was washed.

The stable ATP-analogue β - γ -M ATP ($1\text{--}1000 \mu\text{M}$) as previously reported (Brown et al. 1971) caused a stronger initial contraction than ATP (Fig. 1). But as ATP it dose-dependently inhibited the contraction response to transmural nerve stimulation (Fig. 1).

Adenosine ($1\text{--}1000 \mu\text{M}$) relaxed the isolated preparation. Moreover adenosine dose-dependently depressed the response to transmural nerve stimulation (Fig. 2, 3) and in this respect was more potent than ATP (Fig. 3). Adenosine ($10 \mu\text{M}$) inhibited the nerve-induced contraction to $20.5 \pm 1.4\%$ before and $15.2 \pm 7.2\%$ after $0.5 \mu\text{M}$ (means \pm S.E., $n=15$ and 5 respectively, $P>0.2$). It is unlikely therefore that the inhibition by adenosine reflects interactions with the cholinergic part of neuroeffector transmission.

To test whether ATP inhibited the contraction response secondarily to being metabolized to adenosine, ADA was added to the organ bath.

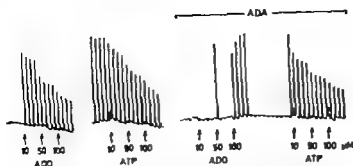


Fig. 2 Isolated rat urinary bladder contraction response to transneuronal nerve stimulation (5 Hz, 0.5 ms, 10 pulses) at 1 min intervals. Adenosine deaminase (ADA 0.2 units/ml) antagonizes the inhibition by adenosine but not that by ATP.

periments. However, while ADA effectively interacted the effect of adenosine (Fig. 2), the action of ATP (Fig. 2) and β - γ -M-ATP (not shown) was present as before ADA. By itself, ADA had no effect on the response to transneuronal stimulation.

The blockade of nerve-propagated responses to transneuronal stimulation by tetrodotoxin (0.3 μ M) and muscle stimulation was accomplished by pulses with long duration (3–25 ms). Tetrodotoxin (100 μ M) inhibited the ensuing contraction to the same extent as the nerve-induced contraction (Fig. 4). Furthermore, the contraction induced by ACh (1–50 μ M) was dose-dependently inhibited by ATP, adenosine and β - γ -M-ATP (Fig.

5). Adenosine was somewhat more potent than the two other substances (Fig. 5).

Adenosine action is known to be terminated both by tissue uptake and by deamination to inosine (Arch & Newsholme 1978). Dipyridamole (0.5–1 μ M) has been reported to reduce adenosine uptake into various tissues by 75–95% (Satchell et al. 1972, Dieterle et al. 1978, Moritoki et al. 1978) and EHNA (1 μ M) inhibited adenosine deaminase activity by 96% in rabbit renal homogenates (Fredholm et al. 1978). In the present experiments, dipyridamole (1 μ M) caused a slight, but significant, inhibition of the contraction response to transneuronal nerve stimulation (10 ± 3 % mean \pm S.E., $n=10$, $P<0.001$) whereas EHNA (1 μ M) was ineffective

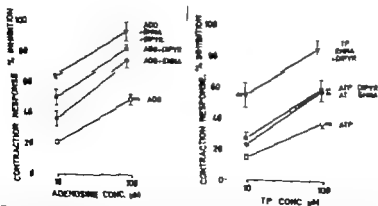


Fig. 3 Isolated rat urinary bladder. Effect of dipyridamole (1 μ M, ●), EHNA (1 μ M, ○) and dipyridamole (1 μ M) + EHNA (1 μ M) (V) on the inhibition by adenosine and by ATP of contraction of responses to transneuronal nerve stimulation (5 Hz, 0.5 ms, 10 pulses) at 1 min intervals. Mean values \pm S.E., number of expts. within parentheses. EHNA or dipyridamole significantly ($P<0.001$) increased the inhibition. EHNA + dipyridamole caused further increases of the inhibition (significant at least at the 5% level, except when comparison was made with dipyridamole + 100 μ M adenosine).

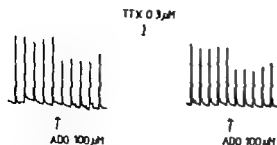


Fig. 4 Inhibition by adenosine of contraction responses to transmural nerve stimulation (5 Hz, 0.5 ms, 10 pulses) and to direct muscle stimulation (5 Hz, 15 ms, 10 pulses) after nerve paralysis by tetrodotoxin.

in this respect. When the two drugs were given together there was no additive effect on the response to electrical stimulation. The inhibitory effect of adenosine (10–100 μ M) on the contraction response to transmural nerve stimulation was markedly increased by dipyridamole or EHNA (Fig. 3). The combination of EHNA and dipyridamole further increased the inhibitory effect of adenosine (Fig. 3). Dipyridamole and EHNA also increased the inhibition by ATP (10–100 μ M) of contraction responses to transmural nerve stimulation and more so when the two drugs were given together (Fig. 3). Noticeably the inhibitory action of β - γ -M ATP (10–100 μ M) on elicited contraction responses was also increased after dipyridamole + EHNA (Fig. 6).

DISCUSSION

Adenosine and ATP dose-dependently inhibited the contraction response to transmural nerve stimulation in the rat (present report) and guinea pig urinary bladder (Dahlén 1979). It has been reported that ATP may increase spontaneous activity and contraction response to intramural nerve stimulation in the guinea pig urinary bladder superfused with a modified Krebs solution containing approximately 0.10 mM magnesium (Burnstock et al. 1978). In our experiments, however, ATP never increased spontaneous activity and the contraction response to transmural stimulation was consistently depressed by ATP, even if the nerve-induced contraction was superimposed on an ATP twitch (Figs 1 and 6). Furthermore, when using a similar modified Krebs solution as Burnstock et al. or even a magnesium free solution, we have observed but

inhibition both in the rat and guinea pig urinary bladder (Dahlén & Hedqvist unpublished).

Purines inhibit the release of neurotransmitter from many autonomically innervated tissues (Hedqvist & Fredholm 1976) but a similar effect in the urinary bladder cannot be evaluated, as the exact nature of the transmission is revealed. However, the present finding that ATP and adenosine depressed the response to both direct muscle stimulation and ACh provides evidence that both ATP and adenosine have a postjunctional effect.

The observed inhibition by ATP of contraction responses is somewhat unexpected in the light of ATP as a proposed excitatory transmitter. Indeed, the inhibition might be due to the metabolism of ATP forming inhibitory degradation products such as adenosine (Brown et al. 1976). However, the present experiments strongly suggest that ATP by itself is inhibitory. Thus, the ATP analogue β - γ -M ATP also inhibited the contraction response to transmural nerve stimulation. In addition, treatment of the tissue with ADP effectively annulled the inhibition by adenosine, but not that by ATP.

It has been proposed that there are several receptors (Burnstock 1976) and it is evident in the rat urinary bladder, one and the same purine, e.g. ATP, may produce both excitation and inhibition, thus suggesting the presence of at least postjunctional purine-sensitive sites. Nevertheless, the main effect of added ATP on nerve-evoked contraction responses appears to be at the inhibitory

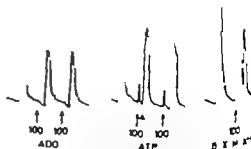
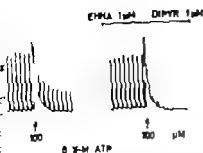


Fig. 5 Inhibition by adenosine, ATP and β - γ -M ATP (added at arrows) of contraction responses to 50 μ M ACh in isolated rat urinary bladder. Preparation 10 min after each ACh-administration.



Isolated rat urinary bladder contraction responses to intravesical nerve stimulation (5 Hz, 0.5 ms, 10 pulses) at intervals. Inhibitory effect of β - γ -M-ATP on bladder contractions by EHNA. Note that contractions are inhibited even when superimposed on the contraction by β - γ -M-ATP.

which could seem to be at variance with the putative "parinergic" excitatory transmission in the bladder.

It is reasonable to assume that dipyrindamole and EHNA at the concentrations used, cause a major inhibition of adenosine inactivation (e.g. Satchell et al 1972; Fredholm et al 1978). Accordingly these enhanced the inhibitory effect of adenosine on nerve-induced contractions. Noteworthy also is that inhibition by ATP was increased after EHNA and/or EHNA, which as suggested by Satchell et al (1972, Arch & Newsholme 1977) might be due to product inhibition of ATP degradation when adenosine accumulates. But, it is likely that such mechanisms contribute to the enhanced inhibition by β - γ -M-ATP after EHNA plus EHNA. It is, however possible both ATP per se and β - γ -M-ATP might be released via the same uptake mechanism as adenosine.

It seems well established that stimulation of adrenergic (Su 1975; Fredholm & Hedqvist 1978), cholinergic (Sulzsky 1975) and non-cholinergic adrenergic (Burnstock et al 1970) nerves causes a release of purines of which adenosine is likely to be a major component (Satchell & Stock 1971; Fredholm & Hedqvist 1978). Stock et al (1978) reported that intravesical stimulation of the superfused guinea pig urinary bladder released ATP but release of other purines was not looked for. Endogenous purines, including adenosine, have been suggested to function as modulators of adrenergic and cholinergic effector transmission (Hedqvist & Fredholm

1976; Sawynok & Jhamandas 1976; Vizi & Knoll 1976; Gustafsson et al 1978). The present finding that ATP and adenosine inhibited nerve-induced contractions in the rat urinary bladder might indicate a modulatory role for purines also in non-cholinergic non-adrenergic transmission. The observation that dipyrindamole by itself inhibited the transmission is consistent with this view. Since purines could derive both from nervous and non-nervous sources the possibility of endogenous purines modulating the non-cholinergic non-adrenergic excitatory transmission in the rat urinary bladder is not critically dependent of whether parinergic nerves exist or not.

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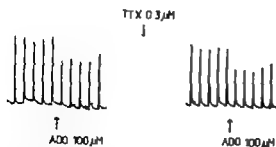


Fig. 4 Inhibition by adenosine of contraction responses to transmural nerve stimulation (5 Hz, 0.5 ms, 10 pulses) and to direct muscle stimulation (5 Hz, 15 ms, 10 pulses) after nerve paralysis by tetrodotoxin.

In this respect. When the two drugs were given together there was no additive effect on the response to electrical stimulation. The inhibitory effect of adenosine (10–100 μ M) on the contraction response to transmural nerve stimulation was markedly increased by dipyridamole or EHNA (Fig. 3). The combination of EHNA and dipyridamole further increased the inhibitory effect of adenosine (Fig. 3). Dipyridamole and EHNA also increased the inhibition by ATP (10–100 μ M) of contraction responses to transmural nerve stimulation and more so when the two drugs were given together (Fig. 3). Noticeably the inhibitory action of β - γ -M ATP (10–100 μ M) on elicited contraction responses was also increased after dipyridamole+EHNA (Fig. 6).

DISCUSSION

Adenosine and ATP dose-dependently inhibited the contraction response to transmural nerve stimulation in the rat (present report) and guinea pig urinary bladder (Dahlén 1979). It has been reported that ATP may increase spontaneous activity and contraction response to intramural nerve stimulation in the guinea pig urinary bladder superfused with a modified Krebs solution containing approximately 0.10 mM magnesium (Burnstock et al. 1978). In our experiments, however, ATP never increased spontaneous activity and the contraction response to transmural stimulation was consistently depressed by ATP, even if the nerve-induced contraction was superimposed on an ATP twitch (Figs 1 and 6). Furthermore, when using a similar modified Krebs solution as Burnstock et al. or even a magnesium free solution, we have observed but

inhibition both in the rat and guinea pig urinary bladder (Dahlén & Hedqvist unpublished).

Purines inhibit the release of neurotransmitters from many autonomically innervated tissues (Hedqvist & Fredholm 1976) but a similar effect in the urinary bladder cannot be evaluated. The exact nature of the transmission is revealed. However, the present finding that ATP and adenosine depressed the response to both direct muscle stimulation and ACh provides evidence that both ATP and adenosine have a postjunctional effect.

The observed inhibition by ATP of contraction responses is somewhat unexpected in the rat urinary bladder, as ATP is proposed as an excitatory transmitter. Indeed, the inhibition might be due to metabolism of ATP forming inhibitory degradation products such as adenosine (Brown et al. 1978). However, the present experiments strongly suggest that ATP by itself is inhibitory. Thus, the ATP analogue β - γ -M ATP also inhibited the contraction response to transmural nerve stimulation. In addition, treatment of the tissue with ADP partially annulled the inhibition by adenosine but not that by ATP.

It has been proposed that there are several receptors (Burnstock 1976) and it is evident in the rat urinary bladder, one and the same purine, e.g. ATP, may produce both excitation and inhibition, thus suggesting the presence of at least postjunctional purine sensitive sites. Nevertheless, the main effect of added ATP on nerve-elicited contraction responses appears to be at the inhibitory site.

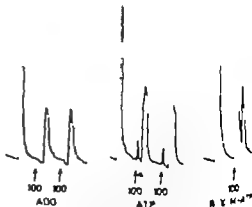


Fig. 5 Inhibition by adenosine, ATP and β - γ -M ATP (added at arrows) of contraction responses to 50 μ V (Δ) in isolated rat urinary bladder. Preparations were washed after each ACh-administration.

Effect of polycythemia on blood flow in working and non working skeletal muscle

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GUSTAFSSON L. APPELGREN L. & MYRVOLD H. E. The effect of polycythemia on blood flow in working and non-working skeletal muscle. *Acta Physiol Scand* 1980 109: 143-148. Received 1 Oct 1979. ISSN 0001-6772. Department of Surgery I and II and Department of Anaesthesiology III, University of Göteborg, Sweden

The effect of acutely induced polycythemia on blood flow and viscosity in the vasodilated vascular bed of working and non-working skeletal muscle was studied. In 12 mongrel dogs anesthetized with thiopental sodium the calf muscle of one hind limb was isolated. Vasodilation was induced either by sciatic stimulation setting the muscle at maximal work or by i.a. infusion of papaverine. Blood flow was measured at different perfusion pressures before and after infusion of 300 ml packed homologous red cells. Blood viscosity *in vitro* was determined as complete viscometer. Apparent viscosity *in vivo* was analyzed by comparing pressure-flow relationships for blood and a reference solution. Polycythemia decreased blood flow by 35% in the non-working muscle but less than 10% in the working muscle at comparable perfusion pressures. Blood viscosity *in vitro* increased by 35% at low shear rates. Apparent viscosity *in vivo* increased by 35% in the non-working muscle but less than 10% in the working muscle. The flow impairment induced by polycythemia was far more pronounced in the non-working skeletal muscle indicating a flow facilitation by the rhythmic muscle contractions. Erythrocyte flow in fact increased in the working muscle after induced polycythemia while decreased in the non-working muscle.

Key words: Skeletal muscle blood flow, exercise, polycythemia, viscosity

ly induced polycythemia is associated with an increase in cardiac output and venous return (Levy & Richardson 1961; Reptig & Merrill 1964; Wense et al. 1964). The change in blood flow induced by polycythemia seems to be approximately equal to changes seen in blood viscosity *in vitro*. The higher oxygen carrying capacity offered by polycythemia is therefore to some degree offset by the decrease in flow due to increased viscosity. In skeletal muscle the blood flow is dependent on metabolic demand and there is a decrease of flow at increasing hematocrits (Levy & Share 1953; Messmer 1972; Whitaker & Winton 1933). The apparent viscosity *in vivo* is however considerably lower than *in vitro* (Djojanzigito et al. 1970; Levy & Share 1953; Whitaker & Winton 1933). Furthermore the effect of hematocrit on apparent vis-

cosity *in vivo* is less pronounced than on viscosity *in vitro* (Levy & Share 1953; Whitaker & Winton 1933). This can be explained by the Fåhræus-Lindqvist effect acting in small vessels and reducing viscosity *in vivo* (Djojanzigito et al. 1970).

These conclusions are based on experiments performed during resting conditions. During physical performance a small increase of hematocrit might, however be of benefit for the capacity of the organism (Ekblom et al. 1977, 1976). During such conditions the advantage of increased oxygen carrying capacity seems to be more important than the disadvantage of increased blood viscosity.

It was the purpose of this study to investigate the effect of polycythemia upon pressure-flow relationships and on apparent viscosity *in vivo* in the isolated working and non-working calf muscle of the dog.

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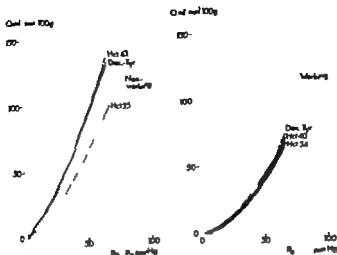


Fig. 3 Pressure-flow relationships for Dextran-Tyrosine solution (—) in the working and vaso-dilated non-working muscle as relation to the pressure-flow relationships for blood as taken from Fig. 2. For clearness spread of data was omitted.

tal volume of red cells transported to the muscles as an indication of oxygen availability was determined at normal hematocrit and after polycythemia by measuring hematocrit by the flow rate (Ogston & co 1961).

Local values for apparent blood viscosity *in vivo* at perfusion pressures are calculated from the flow curves for blood and for syngested Dextran solution. Newtonian solutions with consistency of 2.2 cp assuming the blood and reference viscosities in the calf muscle to be inversely equal to the registered flows at certain perfusion pressure.

Viscosity *in vitro* of whole blood and plasma were determined in Wells-Brookfield synergetic cone-plate rheometer model LVT using 1 ml samples of heparinized blood and plasma. Viscosity measurements made on two samples at each hematocrit and blood was viscometrically with the flow measurements. Hematocrits are made in shear rates of 20, 40, 115

and 230 s⁻¹. Hematocrits were measured in micro-hematocrit tubes.

For statistical analysis the Wilcoxon matched-pairs signed-rank test was used.

RESULTS

After transfusion hematocrit increased in all dogs. Hematocrit increased from 42 ± 1.2 to 55 ± 0.5 (mean \pm S.E.) in the group with non-working muscle and from 40 ± 1.1 to 54 ± 1.1 in the group with working muscle. After acutely induced polycythemia there was a numeric decrease of flow in both non-working and working skeletal muscle. In the non-working group, the flow was 138 ± 6 ml/min/100 g (mean \pm S.E.) at a perfusion pressure of 70 mmHg and normal hematocrit and 100 ± 5.5 ml/min/100 g

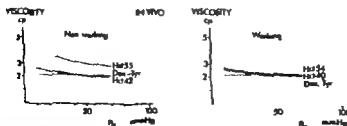


Fig. 4 Average relationship between perfusion pressure and calculated apparent viscosity *in vivo* in the working (—) and vaso-dilated non-working (---) skeletal muscle at normal hematocrit and after induced polycythemia.

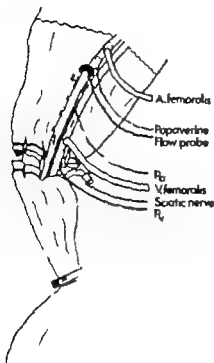


Fig. 1 The isolated calf muscle of the dog.

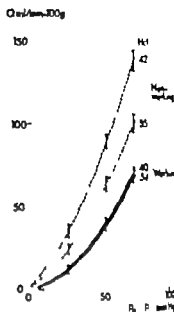


Fig. 2 Pressure-flow relationships in the sedentary and vasodilated non-working (—) skeletal muscle at normal hematocrit and after induced polycythemia. Spread of data is given in 3 representative perfusion pressures of 35, 50 and 70 mmHg (mean \pm SE).

MATERIALS AND METHODS

1 mongrel dogs (body weight 15–17 kg) were anesthetized with i.v. thiopental sodium (15 mg/kg) with repeated doses (2–4 mg/kg) throughout the experiment. The dogs were intubated endotracheally and ventilated with air using a volume controlled respirator. Blood gases were controlled and adjusted throughout the experiment.

The flow measurements were performed on the isolated calf muscle of one hind limb of the dog. The experimental model was in principle (but described in previous studies (Djojosegito et al 1970, Gustafsson et al 1977)) but with some modifications (Fig. 1).

The thigh muscles were divided above the knee joint, as were all vessels except the popliteal artery and vein. The skin was loosened around the calf in order to exclude cutaneous blood flow after which the skin was repositioned thus protecting the preparation. The paw was excluded by a screw clamp just above the ankle joint. The femoral superficial artery was isolated and all side branches were divided except two which were used for pressure registration and drug infusion.

Wide-bore silicized T-tubes were introduced in the femoral artery and vein for intermittent extracorporeal perfusion of the isolated limb with oxygenated Dextran-Tyrod solution (6% Dextran 40 in Ringer's solution) from a pressure bottle connected to the arterial T-tube.

The perfusion pressure was measured with Statham pressure transducers, model P 23, in arterial and venous catheters and was registered continuously on a Grass polygraph, model 7 C. In order to obtain different perfusion pressures an adjustable screw clamp was placed on the artery proximal to the pressure catheter. Blood flow

was measured with an electromagnetic flow meter (Trone model 377) with the flow probe placed on the artery proximal to the screw clamp. Another side branch artery was used for infusion of papaverine.

Polycythemia was induced by transfusion of 50 ml packed homologous red cells into a central vein. Cells are allowed to mix in the animal for about 15 min before new measurements were performed.

In six dogs flow measurements were performed in muscles set at heavy rhythmic exercise by the cut end of the sciatic nerve with square wave from a Grass stimulator. The stimulation duration was 3–5 V, 3–5 ms and 4–6 impulses per second. Flow measurements were performed on the muscles where the vascular bed was vasodilated by a constant i.a. infusion of papaverine (80 mg/min).

Pressure-flow curves were constructed by step adjustments of the screw clamp. The pressure-flow relationships in the working and vasodilated non-working muscles were established at normal hematocrit and polycythemia for perfusion pressures between 35 and 70 mmHg. By adjusting the screw clamp in discrete steps blood flow, expressed in ml per minute per 100 g, was obtained as a function of perfusion pressure. The hematocrit of the screw clamp was adjusted from 40% flow down to complete closure and reopened at about 70 distinct steps altogether maintaining the perfusion pressure constant for 5–10 s at each step. The long-reopening procedure was repeated three to five times and the values of flow and perfusion pressure were plotted on a pressure-flow diagram. Pressure-flow curves were also registered for the oxygenated Dextran-Tyrod solution of constant

obtained by papaverine compared to metabolite-induced vasodilation as demonstrated by Jones et al (1975) who during infusion of adenosine in the exercising muscle saw an increase of flow by approximately 25–30% compared to flow during exercise alone.

Apparent viscosity *in vivo* could be calculated by perfusing the limb with a reference solution and measuring its viscosity (Diosogugito et al. 1970). Since an Tyrode solution behaves as a Newtonian fluid the pressure-flow curve for this perfusate will be a physical description of the vascular bed, i.e. a calibration curve for the biological viscometer. Comparison of flow values for blood and Tyrode solution at identical perfusion pressures gives apparent blood viscosity *in vivo* could be calculated. The apparent viscosity *in vivo* was found to be considerably lower than that measured *in vitro* which is in line with several other studies (Saguto et al. 1970; Levy & Shure 1943; White & Winton 1933). This is probably due to the nature of the Fåhræus-Lindqvist effect in vessels with consequent reduction of apparent viscosity. Calculation of apparent blood viscosity from the very lowest part of the pressure-flow curve was not reliable with the blood flow method used. It was therefore not possible to calculate reduction of viscosity *in vivo* in very low flow conditions described by others (Diosogugito et al. 1970). In comparing flow rates at different perfusion pressures in working and non-working muscle, polycythemia caused a marked impairment of flow in non-working muscle but not in the working muscle. The flow decrease obtained during non-working conditions is of the same magnitude that was evaluated by extrapolation from data by Jones & Richardson (1961) on venous return and by Jones & Shure (1953) on flow in the hind limb. High rhythmic muscle contractions per se produce resistance to flow; the rhythmic muscle contractions will also counteract the disadvantages of increased hematocrit and thereby increased blood viscosity. In fact the oxygen availability increased in the increased hematocrit in the working muscle and decreased in the non-working muscle. The net effect of rhythmic muscle contractions on local blood flow may be due to an increased pressure gradient. Muscular contractions might compress the radius of small veins and might laterally empty them of their content of blood. The pressure of the veins will decrease the mean post-

capillary pressure with a corresponding gain in effective perfusion pressure (Folkow et al. 1970, 1971). If so rhythmic muscle contractions might increase flow velocity in the capacitance wide-bore vessels where flow and shear rate are proportionally low and viscosity is proportionally high especially at increased hematocrit.

Beside this postcapillary mechanism another precapillary mechanism is worth considering. Öberg et al. (1975) demonstrated that, in the pharmacologically dilated vascular bed and with blood as perfusion medium, precapillary resistance and subsequently effective precapillary viscosity were unexpectedly higher than postcapillary resistance and effective postcapillary viscosity. One explanation might be that the higher precapillary resistance found with a corpuscular perfusion medium is due to mechanical hindrance and friction occurring when the corpuscular elements of blood are squeezed through the metarterioles and capillaries. In line with this, it might be argued here that heavily exercising muscle facilitates the passage of red cells by squeezing them through the precapillary and capillary network, thereby preventing an expected increase of effective precapillary viscosity provoked by a denser train of red cells passing at a higher hematocrit.

These tentative mechanisms of flow facilitation by muscular rhythmic work at high hematocrits—one precapillary and one postcapillary—suggest different ways for a small part of the muscular mechanical work to be utilized for blood flow improvement. Even if the mechanisms of the flow improving effects of rhythmic muscle contractions at high hematocrits are not known and merit further investigation the present study clearly shows an increased oxygen availability in the muscular tissue in the polycythemic state. This is in line with earlier findings of benefit of a small increase of hematocrit for the aerobic capacity of the organism during physical performance (Ekblom et al. 1972, 1976).

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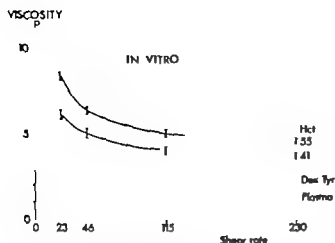


Fig. 5 In vitro viscosity data for whole blood, Dextran-Tyrode solution and plasma at normal hematocrit and after induced polycythemia (mean \pm S.E.)

after induced polycythemia ($P < 0.05$) compared to 70 ± 4 and 66 ± 4 ml min⁻¹ 100 g⁻¹ in the working group (n.s.). At a perfusion pressure of 25 mmHg the flow in the non-working group was 35 ± 3.7 ml min⁻¹ 100 g⁻¹ at normal hematocrit and 24 ± 3.0 ml min⁻¹ 100 g⁻¹ after polycythemia. In the working group the corresponding values were 12 ± 2 and 11 ± 2 ml min⁻¹ 100 g⁻¹ respectively (Fig. 2).

In the non-working group blood flow decreased about 35% after red cell transfusion at corresponding perfusion pressures ($P < 0.05$) with a slightly more pronounced decrease at low perfusion pressures. During exercise there was only a small, non-significant decrease in flow (5–10%) over the whole pressure range after acutely induced polycythemia.

The pressure-flow curves for the Dextran-Tyrode solution in the working and vasodilated non-working muscles are shown in Fig. 3. The pressure-flow curve for the reference solution in the working group is based on values from only 3 dogs since in the additional 3 there were signs of impaired rhythmic contractions and thereby submaximal flow during extracorporeal perfusion. As indicated the pressure-flow curve for the Dextran-Tyrode solution is almost identical with that for blood of normal hematocrit in both working and non-working muscle.

Calculated apparent viscosities in vivo at normal hematocrit and after polycythemia in the working and vasodilated non-working muscles are shown in Fig. 4. Apparent viscosity in vivo after polycythemia was 35% higher compared to normal blood with a tendency to an increasing difference at

lower perfusion pressures in the non-working muscles ($P < 0.05$). In the working muscle the η of apparent viscosity in vivo was less different (n.s.).

The in vitro whole blood viscosity measurements revealed highly significant differences between normal and polycythemic conditions at all shear rates (Fig. 5). At shear rates of 23 s⁻¹ the η of whole blood viscosity was about 35% higher in polycythemia or after the perfusion procedure. No change was seen in plasma viscosity.

Minute flow of red cells decreased by 35% in polycythemia in the non-working muscle but increased about 25% ($P < 0.05$) in the working muscle (Fig. 6).

DISCUSSION

The experiments were performed in a well-defined vascular bed where vessel geometry and because of smooth muscle activity were not changing, thus inducing a pronounced vasodilation of the bed. Vasodilation was induced either mechanically by heavy rhythmical exercise or by i.v. infusion of papaverine. At normal hematocrit blood flow in the papaverine-dilated non-working muscle exceeded flow in the exercise-dilated working muscle about 100% indicating that the twitching contractions of the muscles provide a mechanical resistance to flow. The flow difference is probably to some degree due to an additional vasodilation

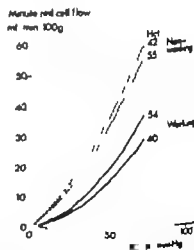


Fig. 6 Effect of polycythemia on minute volume of red cell flow into working (—) and vasodilated non-working (---) skeletal muscle.

Effects of physical training on skeletal muscle metabolism and ultrastructure in 70- to 74-year-old men

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ÖRLANDER, J. & ANIANSSON, A. Effects of physical training on skeletal muscle metabolism and ultrastructure in 70 to 74-year-old men. *Acta Physiol Scand* 1980, 109, 149-154. Received 5 Oct. 1979. ISSN 0001-6772. Department of Animal Nutrition, Swedish University of Agricultural Sciences, Uppsala, and Department of Rehabilitation Medicine, Sahlgren Hospital, Göteborg, Sweden.

The effects of 12-week program of physical training on skeletal muscle (vastus lateralis) characteristics in 3 old men were investigated. Heart rates during submaximal bicycle exercise were decreased after training, indicating an improved cardiovascular function. As judged from enzyme activity measurements, the anaerobic capacity as well as the mitochondrial oxidative capacity were increased by the training. Fatty acid oxidation capacity remained unchanged while the glycolytic potential tended to be increased, suggesting somewhat different patterns of adaptation as compared to that seen in young subjects. Volume fractions of mitochondria and lipid droplets were unchanged. In training, supporting the view that in old men, increases in oxidative capacity take place within the existing mitochondrial volume. It was concluded, that the aging human skeletal muscle remains trainable and that the training response is similar but possibly not identical to that seen in younger age groups.

Key words: Aging, muscle metabolism, muscle ultrastructure, training.

well-established regular decline in maximal performance capacity and maximal oxygen uptake with aging (e.g. Åstrand 1960, Åstrand et al. 1973, Borg 1973, Aström et al. 1973, Kærsting 1978) was previously thought of as an inevitable process (for ref. see Stamford 1972). However, numerous studies have shown that regular physical training produces significant muscular adaptations in middle-aged and persons (Beastad 1963, Sahlin et al. 1969, Davies 1970, Terjung et al. 1973, Adams & de Kærsting et al. 1974, Llesien et al. 1973, Clark et al. 1976, Suominen et al. 1977a, 1977b, & Shepherd 1978, for other ref. see Beasley).

In general, relative improvements similar to those in young subjects have been observed. In absolute terms, however, the improvement is small in old age, which means that the age decline cannot be offset by training (e.g. Sahlin et al. 1969).

Considerably less is known about the effects of training on skeletal muscle in old age. Llesien et al. (1975) and Suominen et al. (1977a, 1977b) observed increases in enzyme activities of energy metabolism with training in 55 to 70-year-old subjects. In a recent cross-sectional study (Örlander et al. 1978) we observed no age-related decrease in enzyme activities in sedentary men (age 22-65) and found elevated activities in an older relatively active group. This seemed to indicate a persisting trainability in old men, but with a possibly different pattern of adaptation when compared with younger subjects (Örlander et al. 1977, 1978).

The present study was designed to further examine the response of the metabolism of the aging human skeletal muscle to physical training. It is part of a larger study where effects on strength and muscle morphology are analyzed (Aniansson et al. 1980).

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Enzyme activities in vastus lateralis before and after training

means \pm S.E. for 5 subjects. For full names of enzymes, see Material and Methods

Enzyme

| PFK | LDH | HAD | CS | Cyto |
|--|-----------------|-----------------|-----------------|--|
| $\mu\text{mol min}^{-1} \times (\text{g wet wt})^{-1}$ | | | | $\mu\text{mol O}_2 \times \text{min}^{-1} \times (\text{g wet wt})^{-1}$ |
| 16.2 \pm 3.0 | 38.6 \pm 9.3 | 8.00 \pm 1.74 | 8.43 \pm 1.33 | 4.91 \pm 0.52 |
| 28.4 \pm 4.9 | 57.8 \pm 11.9 | 6.88 \pm 0.82 | 8.87 \pm 0.25 | 6.43 \pm 0.53 |
| >0.05 | <0.01 | >0.30 | >0.70 | <0.01 |

to 46% after training. These values seem but higher than the 32% observed in the of more active old men, but no definitive ones can be drawn from these limited obser-

monogram (Åstrand 1960) suggests a substantial increase in predicted maximal oxygen uptake. The extent of this increase is somewhat uncertain due to low post-training heart rates, and the high age of the subjects.

In addition the strength of the right knee-extensor muscles tended to be increased after training (data not shown). In a larger group of subjects this increase was significant (Andarsson et al. 1980).

Before training, LDH activities were lower and those of HAD higher while PFK, CS and cytochrome were in the range previously observed in younger age groups (Orlander et al. 1978) (compensation for the differing assay temperatures was made assuming a Q_{10} of 2). The very low LDH activities are, however, in agreement with the findings of Åström et al. (1977) in 65 to 82-year-old men who both during and 4-6 months after acute bacterial infec-

DISCUSSION

Results of the present investigation indicate that training produces similar effects in old as in young men, including improved cardiovascular capacity as well as metabolic adaptations in skeletal muscle.

In agreement with other studies (for ref. see introduction), the cardiovascular function was improved by the present training regimen, as evidenced by the reduced heart rates during submaximal work. Application of the Åstrand-Åstrand

Ultrastructural parameters in vastus lateralis muscle fibres before and after training

Table 1. Ultrastructural parameters in vastus lateralis muscle fibres before and after training. Data from a group of active men, aged 65-75 are included (from Orlander et al. 1978). Values are \pm S.E.

| | | Mitochondria in fibrillar space | | | | Mitochondria in subsarco-meral space | | | Lipid droplets |
|--------|---|-----------------------------------|-----------------------------------|--|-----------------------------------|--------------------------------------|-----------------------------------|--|-----------------------------------|
| | | Volume fraction (%) | Number μm^{-2} | 100 \times Mean volume μm^3 | | Volume fraction (%) | Number μm^{-2} | 100 \times Mean volume μm^3 | Volume fraction (%) |
| Before | 4 | 2.38 ± 0.29 | 1.81 ± 0.48 | 0.88 ± 0.13 | 2.08 ± 0.34 | 24.1 ± 1.7 | 9.79 ± 2.63 | 3.22 ± 0.95 | 0.43 ± 0.12 |
| After | 4 | 2.70 ± 0.31 | 2.07 ± 0.64 | 0.95 ± 0.18 | 1.89 ± 0.27 | 20.6 ± 2.2 | 7.92 ± 0.94 | 2.54 ± 0.26 | 0.43 ± 0.17 |
| Before | 7 | 2.42 ± 0.35 | 1.36 ± 0.79 | 0.83 ± 0.14 | 2.63 ± 0.60 | 13.1* ± 4.4 | 7.78* ± 2.66 | 2.73* ± 0.69 | 0.48* ± 0.16 |
| After | 7 | >0.40 ± 0.20 ± 0.30 | >0.20 ± 0.30 ± 0.10 | >0.50 ± 0.60 ± 0.60 | >0.20 ± 0.60 ± 0.10 | >0.10 ± 0.10 ± 0.20 | >0.60 ± 0.70 ± 0.90 | >0.60 ± 0.70 ± 0.80 | >0.90 ± 0.50 ± 0.90 |

MATERIAL AND METHODS

Subjects 5 men volunteered to participate in the study. None showed any symptoms of cardiovascular or locomotor disease on medical examination. Each subject was fully informed of the procedure and possible risks of the experiments before giving his consent. The men were all pensioners with various previous occupations and had never participated in any systematic physical training. They currently engaged only in light physical activities such as going for a walk and were therefore considered sedentary. Their age, height and weight at the start of the study were 71 ± 1 (70–75) years, 173 ± 3 (166–185) cm and 68 ± 4 (62–85) kg, respectively (means \pm S.E. ranges within brackets). All of them but one were non-smokers.

Training regimen The training was performed under supervision during 45 min 3 times a week for 12 weeks. Each training workout started with general warm-up exercises such as walking or jogging. Then various static and dynamic exercises using body weight and not special equipment as resistance were performed with the main aim to increase muscular strength but also the capacity for prolonged work. The work load during training sessions was estimated to correspond to no more than 70% of the predicted maximal oxygen uptake and was unchanged during the training period. The training was considered to be moderately intense for this age group. None of the 5 subjects experienced any medical complications related to the training. Before and after the training period the heart rate response to two submaximal loads (50 and 100 W) on the bicycle ergometer was studied. One subject was however unable to complete the 100 W test before training.

Muscle biopsies A needle technique (Bergström 1962) was used to obtain samples from the right vastus lateralis muscle. One part of the sample was trimmed, oriented, mounted in an embedding medium, frozen in isopentane cooled by liquid nitrogen and stored at -80°C for subsequent histochemistry. Another part was immediately frozen in liquid nitrogen and stored at -80°C pending enzyme activity determinations. A third part was prepared for electron microscopy.

Histochemistry Transverse sections (10 μm) were cut with a cryotome and stained for myofibrillar ATPase after alkaline (pH 10.3) or acid (pH 4.4 and 4.6) preincubation (Padykula & Herman 1955; Outh & Samaha 1969; Brooke & Kaiser 1970). Fibres were classified as type I (slow twitch) or types II A–C (fast twitch) (Engel 1962; Brooke & Kaiser 1970; Dubowitz & Brooke 1973). The mean number of counted fibres per biopsy was 319 (before) and 324 (after).

Enzyme assays Homogenates were prepared and assayed as described previously (Örlander et al 1977) except that assays were run at 30°C . The 5 studied enzymes chosen to represent the major pathways in energy metabolism were phosphofructokinase (PFK, E.C.2.7.1.11), lactate dehydrogenase (LDH, E.C.1.1.1.27), 3-hydroxyacyl-CoA dehydrogenase (HAD, E.C.1.1.1.35), citrate synthase (CS, E.C.4.1.3.7) and cytochrome oxidase (cytochrome oxidase, E.C.1.9.3.1). The assay methods used were those of Shoen & Boyer (1964) for PFK, Bass et al (1969) for LDH and HAD, Sere (1969)

for CS and Wherret et al (1969) for cytochrome oxidase, estimated according to Lowry et al (1951).

Electron microscopy Small pieces of muscle were fixed for several hours at $0-4^\circ\text{C}$ in 4% buffered glutaraldehyde, pH 7.4, washed and fixed in 1% veronal-buffered osmium tetroxide for one hour. Subsequent treatment and embedding followed the procedure previously described (Örlander 1977). The calculation of mitochondrial membrane volume involves assumptions regarding cristae shape and size distribution (Weibel 1969). The obtained values are only rough estimates. For various reasons no post-training analysis was possible in one subject. Therefore only 4 subjects are included in the ultrastructural results. The pre-training values of subject were similar to those of the others.

Statistics Intraindividual and inter-group differences were evaluated by means of the paired and unpaired *t*-tests, respectively. A *P* value of less than 0.05 was accepted as significant.

RESULTS

The training program resulted in significant changes in heart rates at both studied submaximal loads. At 50 W a decrease from 110 ± 5 (mean \pm S.E.) to 90 ± 3 beats \times min $^{-1}$ ($n=5$, $P<0.01$) was observed. A similar decrease occurred at 100 W from 119 ± 5 to 105 ± 9 beats \times min $^{-1}$ ($n=4$, $P<0.01$).

The percentage type I fibres in the vastus lateralis was $51 \pm 4\%$ (mean \pm S.E.) before and after training ($P>0.20$). A slight tendency to an increase in type II A fibres ($P>0.10$) was observed from 38 ± 5 to $48 \pm 3\%$ ($n=4$), whereas the proportions of types II B and II C fibres were unaffected (12 ± 6 to $11 \pm 4\%$ and 0.5 ± 0.5 to $0.8 \pm 0.6\%$, respectively).

The activities of HAD and CS were unaffected by training, while LDH (49%) and cytochrome oxidase increased significantly (both $P<0.01$). Table 1 showed a next-to-significant increase ($P<0.10$) with training. The muscle protein content did not change.

As shown in Table 2 the volume fraction of mitochondria and mean volume of skeletal muscle mitochondria were unaffected by the training in the fibrillar and the subarcolemma regions. The same was true for the volume fraction of myofibrils. Furthermore these ultrastructural parameters showed no significant differences when compared with the corresponding data of a group of active 66 to 76-year-old men (Örlander et al 1977) as shown in Table 3. Lipofuscin occurred in the studied subarcolemmal regions before and after training.

tion had only half the LDH activities of young men in the same situations. Low LDH activity may be the consequence of a sedentary way of life as suggested earlier in younger men (Bass et al 1976, Örlander et al 1977) but aging processes may also be involved. As to the high HAD activities, no obvious explanation can be given. The plasma level of free fatty acids tends to increase with age (O'Sullivan et al 1971) and it is conceivable that substrate induction phenomena might play a role.

LDH was seen to increase 49% with training in the present subjects, suggesting an elevated anaerobic capacity. This observation is in accordance with previous findings in old (Liesen et al 1975, Suominen et al 1977a) and young (Örlander et al 1977) sedentary men. The 31% increase in cytochrome activities indicates that the mitochondrial oxidative capacity was augmented after training. This agrees with other studies in old subjects (Liesen et al 1975, Suominen et al 1977a, 1977b) as well as middle aged and younger groups (Varanuskas et al 1970, Morgan et al 1971, Gollnick et al 1973, Bergman et al 1973, Klessling et al 1974, Henriksson 1976, Salin et al 1976, Bylund et al 1977, Örlander et al 1977). CS and HAD, however, remained unchanged with training, suggesting that the fatty acid oxidation capacity was not markedly influenced. On the other hand, PFK tended to be increased after training. This agrees with the high PFK activities found in a group of active old men (Örlander et al 1978) and these findings collectively suggest an increased glycolytic capacity with training. It thus seems possible that the training response in old men differs somewhat from that in young men with regard to substrate preference. The reason for such a difference would not be very clear, but one contributing factor might be the elevated blood glucose in the aged (O'Sullivan et al 1971) which might be utilized as a primary substrate during exercise. The unchanged activity of CS indicates that the citric acid cycle has adequate capacity for supporting an increased aerobic glycolysis. In fact, the citric acid cycle capacity is known to be closely geared to fatty acid oxidation capacity (Hochachka et al 1977). A consequence of increased carbohydrate utilization during exercise is a more rapid depletion of muscle and liver glycogen which reduces the endurance (cf. Fitts et al 1975).

The increased oxidative capacity was not accompanied by a change in the volume fraction of mitochondria. Furthermore, no significant differ-

ences were seen when compared with active old men (Table 2). This supports the originally put forward by Kjaerling et al (1976) in middle-aged and older persons, i.e. oxidative capacity takes place within the mitochondrial volume, in contrast to the young men where the mitochondrial volume increases with training (Kjaerling et al 1976, Örlander et al 1977, Bylund et al 1977).

In conclusion, the results of the present study indicate that the aging human subject remains trainable at least till the age of training response is of a similar magnitude, possibly of a slightly different nature, compared with that seen in young age. Fark adaptation takes place also in the early system. Thus, the aged benefit from training in a similar way as the young.

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Effects of metabolic inhibitors on the efflux of 5-hydroxytryptamine from pancreatic β -cells

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GYLFE, ERIK. Effects of metabolic inhibitors on the efflux of 5-hydroxytryptamine from pancreatic β -cells. *Acta Physiol Scand* 1980, 109: 155-161. Received 11 Oct. 1979. ISSN 0001-6772. Department of Histology Biomedicum, University of Uppsala, Sweden.

The influence of glucose and metabolic inhibitors on the efflux of tritiated 5-hydroxytryptamine (5-HT) was studied in perfused β -cell-rich oblique mouse islet loaded with trace amounts of 3 H-5-hydroxytryptophan. Glucose stimulated exocytotic release of 5-HT but the effect was rapidly inhibited by 2,4-dinitrophenol, anisoycin A or N-ethylmaleimide. This inhibition was followed by a marked stimulation of 5-HT efflux. The latter phenomenon was reversible when 2,4-dinitrophenol was used but appeared irreversible with anisoycin A or N-ethylmaleimide. The results show that the maintenance of 5-HT within the β -cell depends on energy. It is suggested that both inhibitory and stimulatory effects of 5-HT on insulin secretion depend on release of calcium from the secretory granules after short-circuiting a proton pump across the granule membrane.

Key words: Metabolic inhibitors, secretions, insulin storage, insulin granules

Insulin-releasing β -cells of mice take up both 5-hydroxytryptamine (5-HT; Hellman et al. 1972b; Key & Feldman 1977; Gylfe 1978; Lindström and its precursor 5-hydroxytryptophan (5-HT; Gylfe et al. 1973; Gylfe 1978), the latter being converted to 5-HT (Cagelli et al. 1964; Ark 1971; Tjälve 1971; Gylfe et al. 1973). With different techniques have provided evidence for a granular location of 5-HT in β -cells (Jean-Eichenverry & Zieher 1968; Ekström et al. 1971; Hellman et al. 1972b; Gylfe 1978). and other biogenic amines have been reported to have different effects on insulin secretion some of which may be related to an interaction with calcium within the β -granule (Ericson et al. 1977). The standing of how 5-HT can affect insulin secretion would require knowledge about the systems involved in amine accumulation in the islets. Studies of 5-HT uptake have shown this to be energy-dependent in the β -cells (Lindström 1978). It is evident from the present experiments with metabolic inhibitors that also the release of 5-HT already present within the islet depends on energy. It is suggested that biogenic amines can both inhibit and stimulate insulin

secretion by releasing calcium from the secretory granules.

MATERIALS AND METHODS

Chemicals. DL-5-Hydroxytryptophan (3 H-5-HTP) was obtained from the Radiochemical Centre, Amersham, England. Sigma Chemical Company, St. Louis, Mo., USA supplied N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), bovine serum albumin (fraction V), anisoycin A, 2,4-dinitrophenol and N-ethylmaleimide. The monooxygenase inhibitor N-methyl-N-2-propargylbenzylcarbazole (Pargyline) was a gift from Abbott Laboratories, Chicago, USA, whereas di-methylsulfoxide was supplied by Mallinckrodt Chemical Works, St. Louis, Mo., USA. Other reagents were of analytical grade; distilled and deionized water was used.

Medium. Unless otherwise indicated a Krebs-Ringer medium containing 20 mM bicarbonate, 20 mM Hepes and supplemented with 1 mM Pargyline and 1 mg/ml albumin was used (Gylfe 1978).

Animals and isolation of islets. Adult ob/ob mice were taken from non-inbred colony and used as sources of pancreatic islets containing more than 90% β -cells (Hellman 1965). The animals were starved overnight before they were killed by decapitation under ether anaesthesia. The pancreas was rapidly excised and immersed in medium lacking albumin and Pargyline but supplemented

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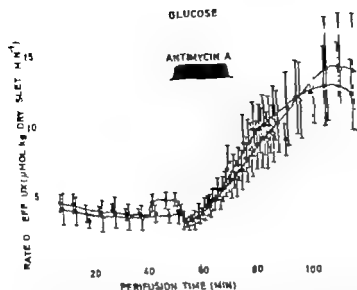


Fig. 2 Effect of antimycin A on 5-HT efflux from pancreatic islets. After loading for 120 min with $16 \mu\text{M}$ (^{3}H)-5-HTP in the presence of 20 mM glucose and 1 mM Pargyline the islets are raised and perfused with medium containing 1 mM Pargyline 0.1% dimethylsulfoxide and either 3 mM glucose throughout (O) or 3 or 20 mM glucose (●) as indicated by the heights of the upper solid bar. Antimycin A ($10 \mu\text{M}$) was added to the perfusion medium as shown by the lower solid bar. Each point represents the mean of 4 observations \pm S.E.

occurred in the presence of both 3 and 20 mM glucose about 6 min after addition of 2,4-dinitrophenol or antimycin A and about 10 min after onset of N-ethylmaleimide. Whereas the late inhibitory effect of 2,4-dinitrophenol on 5-HT efflux is reversed approximately 6 min after removal of the inhibitor, stimulation evoked by antimycin A or N-ethylmaleimide appeared irreversible. Although the onset of the late N-ethylmaleimide effect was comparatively slow it was followed by a rapid increase in the efflux rate which reached a maximal level twice as high as that observed with antimycin A.

DISCUSSION

Cell-rich pancreatic islets from ob/ob mice accumulate both 5-HT (Hellman et al. 1972b; Doney & Feldman 1977; Gyllé 1978) and its precursor 5-HTP (Gyllé et al. 1973; Gyllé 1978). A qualitative microchromatographic procedure revealed that more than 90% of the radioactivity was derived as 5-HT after loading of islets in the presence of radioactive 5-HTP (Gyllé et al. 1973). It agrees in different species with ultrastructural his-

tochemistry (Umm-Etcheverry & Zieher 1968) autoradiography (Ekholm et al. 1971) and subcellular fractionation of islet homogenates (Hellman et al. 1972b) have suggested that 5-HT is located to the secretory granules of the pancreatic β -cell. In accordance with such a location a close relationship was observed between 5-HT efflux and insulin secretion (Gyllé 1978). Genuinely amine-secreting cells like chromaffin cells and neurons are thus not the only cells able to concentrate amines. Indeed this phenomenon seems to be general in cells producing polypeptide hormones. Three different mechanisms have been suggested to explain the amine-accumulating ability of granules. The amine could be taken up and concentrated by a carrier-mediated process coupled to ATP hydrolysis (Krischner 1962; Carlsson et al. 1962). Accumulation could also result from the formation of a strong intragranular storage complex between the amine and ATP resulting in amine entry along its concentration gradient (Bernets et al. 1969a-c). The third and most recent proposal involves ATP-dependent generation of an acidic intragranular space and amine entry along the electrochemical gradient (Johnson et al. 1978; Phillips 1978; Phillips & Allison 1978).

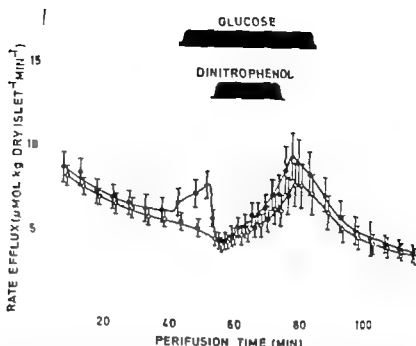


Fig. 1 Effect of 4-dinitrophenol on 5-HT efflux from pancreatic islets. After loading for 120 min with 16 μ M (1H)5-HTP in the presence of 20 mM glucose and 1 mM L-arginine the islets were rinsed and perfused with medium containing 1 mM L-arginine and either 3 mM glucose throughout (O) or 3 or 20 mM glucose (●) as indicated by the height of the upper solid bar. 4-Dinitrophenol (0.3 mM) was added to the perfusion media as shown by the lower solid bar. Each point represents the mean of 4 observations \pm S.E.

with 3 mM glucose. In each experiment 20 islets were microdissected from a single pancreas (Hellerström 1964).

Loading and rinsing. The islets were divided into two groups which were incubated for 120 min at 37°C in 100 μ l medium containing 16 μ M (1H)5-HTP (3700 Ci/mole) and 20 mM glucose. After loading with radioactivity the islets were rinsed twice for 5 min at 37°C in 5 ml non-radioactive medium containing 3 mM glucose.

Perfusion. The two groups of rinsed islets were transferred to 10 μ l perfusion chambers and perfused for 120 min at a flow rate of about 40 μ l/min. A fraction collector was used to obtain 1 or 5 min fractions. In one of the perfusion chambers the medium glucose concentration was maintained at 3 mM and in the other it was raised from 3 to 20 mM between 40 and 80 min. The test substances 4-dinitrophenol (0.3 mM), N-ethylmaleimide (0.1 mM) and antimycin A (10 μ M) were added to both chambers between 50 and 70 min. Antimycin A was added from a stock solution in dimethylsulfoxide maintaining the final concentration of dimethylsulfoxide at 0.1% throughout the experiment.

Weighing of islets and measurement of radioactivity. After perfusion the islets were freeze-dried (-40°C , 0.1 Pa) overnight and weighed on a quartz fibre balance (Lowry 1953). Samples of each fraction (15 μ l) and 100-fold dilutions (15 μ l) of the loading media were diluted in 2 ml toluene Aquasol (1:4 v/v; NEN Chemical GmbH Dreieich F.R.G.). Radioactivity was measured in a Packard 3375 liquid scintillation spectrometer.

Presentation of results. The efflux data (means \pm S.E.)

were expressed as mmol 5-hydroxytryptophan equivalents per kg dry islet assuming that the radioactivity of 5-HT was the same as that of 5-HT loading medium.

RESULTS

The effects of glucose and the metabolite 2,4-dinitrophenol, antimycin A and N-ethylmaleimide on 5-HT efflux are shown in Fig. 1. When the glucose concentration was raised to 20 mM at 40 min of perfusion there was enhanced efflux of 5-HT. Statistical analysis of experiments shown in Figs 1-4 revealed that the effect was significant at the $P < 0.05$ and 1% levels respectively, whereas statistical significance was not reached in the experiments shown in Fig. 5. Exposure of the islets to 0.3 mM 4-dinitrophenol (Fig. 1) or 10 μ M antimycin A (Fig. 2) immediately inhibited glucose-stimulated 5-HT efflux, whereas inhibition was delayed approximately 10 min when 0.1 mM N-ethylmaleimide (Fig. 3) was added to the perfusion medium. The inhibition of glucose-stimulated efflux was followed by pronounced stimulation of 5-HT washout. The

test loading conditions (Gyffe 1978). When into account that more than 90% of the activity in the efflux medium represents 5-HT (1978) it seems justified to express the reverse efflux in terms of μ moles 5-HT assuming no specific activity as in the loading medium was used throughout the experiments. This monoamine oxidase inhibitor enhances accumulation of 5-HT in the islets and consequently facilitates the subsequent efflux studies (1978).

metabolic inhibitors tested accelerated 5-HT after first having reversed glucose-stimulated efflux. The early effect simply follows from energy-dependence of exocytosis since glucose-stimulated 5-HT efflux can be attributed to loss of β -granules (Gyffe 1978). The late effect suggests that the retention of 5-HT taken up by intact β -cells depends on a sustained energy action (though the time-sequence indicates that process is less sensitive than the exocytosis mechanism). Thus if there is a mechanism for 5-HT binding within the β -granules, which is absent it has very low capacity. The lack of permanent binding of the amine is also indicated by the gradual loss of 5-HT from preloaded in the absence of metabolic inhibitors (Gyffe 1978).

The present data do not allow definite views about which of the above-mentioned hypothetical mechanisms accounts for amine accumulation in the β -granules. If a carrier is involved the transport in the β -cells is probably does not fit the results obtained. Available data on the uptake of 5-HT (Lindström 1978) and efflux (unpublished data) indicate that at the high level of 5-HT present in the β -cells after loading, the fluxes can be expected to largely represent

the relatively rapid effect of the mitochondrial β -oxidation pathway A on 5-HT efflux suggests that cytoplasmic level of ATP is of significance for raising the intragranular 5-HT concentration. The most attractive alternative therefore seems to be amine accumulation depending on an acidic granular space. The presence of a low pH in the interior of the β -granule sac might actually be fundamental for maintaining the crystalline form of the stored insulin. Previous studies (Gyffe 1978) indicated that the stored insulin complex is sensitive to pH exhibiting its maximal stability at pH 6.0 (Coore et al. 1969; Howell et al. 1969).

However the intracellular pH was estimated to be no less than 7.03 when measuring the uptake of C-labelled 5,5-dimethyl-2,4-dioxo-2,4-dione into microdissected islets of ob/ob mice (Hellman et al. 1972a). 2,4-Dinitrophenol can consequently be expected to stimulate 5-HT efflux not only by uncoupling of the oxidative phosphorylation but also by short-circuiting the proton pump in the β -granule membrane. Also N-ethylmaleimide could preferentially affect the secretory granules by inhibiting a proton-translocating ATPase. It has been shown in chromaffin cells that this sulphydryl reagent inhibits granule ATPase at concentrations lower than those required to inhibit ATPases from other subcellular fractions (Krahnert et al. 1966).

Depending on species, concentration of glucose and phase of secretion, 5-HT has been reported to inhibit (Lernmark 1971; Feldman & Lebowitz 1972; Gagliardino et al. 1974) stimulate (Gagliardino et al. 1974; Federspiel et al. 1974) or lack effect on the release of insulin (Malaisse 1972). Biogenic amines may affect insulin secretion by acting both at intra- and extracellular sites (Woods & Porte 1976). The effect of extracellular amines may be due to an interaction with calcium in the β -granules (Ericson et al. 1977) an idea compatible with the observation that 5-HTP and 5-HT influence the uptake of intracellular ^{45}Ca in the β -cells (Lindström & Sehlin 1979). The β -granules contain an exchangeable pool of calcium subject to glucose regulation (Hellman et al. 1979). The present data show that 5-HT exhibits a similar sensitivity to metabolic inhibitors as previously reported for glucose-stimulated β -cell calcium (Hellman 1979). Indeed even the Ca^{2+} uptake into the granules may occur in exchange for protons (Hellman et al. 1979). It was suggested that an enhanced concentration of calcium in the submembrane space stimulated insulin secretion only when a certain amount of calcium is present within the granule sac (Hellman et al. 1979). Depending on the calcium content in the granules biogenic amines could thus be expected to exert both inhibitory and stimulatory effects on insulin secretion, the common denominator being release of calcium from the granules after short-circuiting the proton gradient with the amine.

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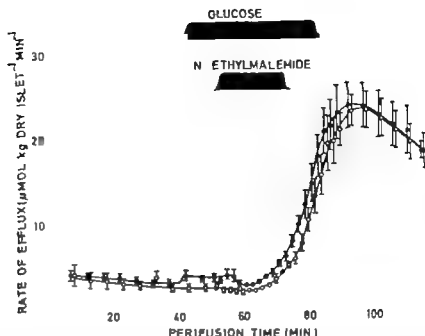


Fig. 3 Effect of N-ethylmaleimide on 5-HT efflux from pancreatic islets. After loading for 120 min with $16 \mu\text{M}$ (PH)5-HTP in the presence of 20 mM glucose and 1 mM Pargyline the islets were rinsed and perfused with medium containing 1 mM Pargyline and either 3 mM glucose throughout (O) or 3 or 20 mM glucose (●) as indicated by the height of the upper solid bar. N-ethylmaleimide (0.1 mM) was added to the perfusion media as shown by the lower solid bar. Each point represents the mean of 4 observations \pm S.E.

Aberer et al 1978). None of these alternatives have been ruled out and combinations between them have actually been proposed. Johnson & Scarpa (1979) favour a carrier-mediated process energized by the electrochemical gradient.

The β -granules differ from chromaffin granules and synaptic vesicles from neurons in not having amines as the major secretory product. Exposure of the adrenal medulla to metabolic inhibitors result in an inhibited release of catecholamines (Rubin 1969, 1970) indicating that maintenance of the amine within the cell might depend on factors other than the energy requiring uptake alone. The presence of more than one process determining the storage of amines in granules is suggested also from the specific storage of one amine in the chromaffin cell or in neurons as opposed to the non-specific accumulation of several amines into the secretory granules of these cells (Shore 1972). In the pancreatic β -cell energy is required for the uptake of 5-HT (Lindström 1978). However nothing is known about the energy requirements for maintaining amines already present in the β -granules.

The mechanisms involved in β -granule storage of

5-HT have now been studied by monitoring efflux from preloaded islets during exposure to metabolic inhibitors. Inhibitors were selected on the basis of their known effects on metabolism and established effects on the β -cell function at concentrations employed. Both antimycin A, a blocker of respiration and 2,4-dinitrophenol, an uncoupler of the oxidative phosphorylation, inhibited the glucose stimulated insulin release (Carr-Randle 1964, George et al 1971). A permanent increase of the plasma membrane in terms of surface area has been observed after exposing rod-islets from *ob/ob* mice to $10 \mu\text{M}$ antimycin A for 60 min (Hellman et al 1973). N-ethylmaleimide was taken as representing a non-penetrating sulphhydryl reagent. When the β -cell type of islets are exposed to 0.1 mM N-ethylmaleimide both the oxidation of glucose and glucose stimulated insulin release are markedly suppressed (Hellman 1979).

The endogenous content of 5-HT in mouse islets has been estimated to approximately 15 μg per wet weight (Hansen & Hedeskov 1977) which is only about 5% of the amount accumulated

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Serotonin metabolism in neonatal rat brain during asphyxia and recovery

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Neonatal rats were exposed to 20 or 30 min of total or partial oxygen deprivation. During asphyxia and subsequent recovery the endogenous levels of tryptophan and 5-hydroxytryptamine (5-HT, serotonin) were monitored. The activity of tryptophan hydroxylase, the first and rate limiting enzyme in the 5-HT synthesis pathway, was studied *in vivo* by measuring the accumulation of 5-hydroxytryptophan (5-HTP) after inhibition of aromatic L-amino acid decarboxylase with NSD 1015. During asphyxia there was a decrease in tryptophan hydroxylase activity in the whole brain and various brain regions studied. The levels of tryptophan, 5-HTP and 5-HT all increased after 30 min of recovery from asphyxia. In the whole brain, 5-HTP and 5-HT levels were normal 2 h after asphyxia while tryptophan levels normalized more slowly to reach control values after 6 h. In the regional brain study the 5-HTP levels returned quickly to control levels after asphyxia in the striatum and midbrain but not in the brainstem and hemispheres regions. The whole brain 5-HTP and 5-HT levels did not differ from controls 24 and 48 h after the asphyxia. Although the neonatal nervous system exhibits great resistance to asphyxia, the metabolism of the neurotransmitter 5-HT is affected already during a short period of asphyxia and subsequent recovery. As 5-HT is described important neurotransmitter functions, this might be relevant to the neurological sequelae of neonatal asphyxia.

Key words: Experimental study, neonatal asphyxia, 5-HT, CNS neurotransmitter.

5-hydroxytryptamine (5-HT) has been ascribed a number of functions as neurotransmitter in the central nervous system as for example, control of sexual behaviour, induction/repression of sexually active behaviour, central regulation of body temperature and participation in a number of psychic functions (Lewin 1964; Reid *et al.* 1968; Jouvet 1969). Recent studies have demonstrated that 5-HT is present in the brain relatively early in the ontogenetic development. By means of histochemical techniques, 5-HT can be visualised in various cell bodies in the raphe nuclei as well as in the nerve terminals at the base of the midbrain already in the second trimester of the rat (Olsson *et al.* 1972). The development of the serotonergic system is from this point accelerating to reach adult state in the 5th or 6th postnatal week in the rat (Benoit & Guzman 1965; Schmidt & San-

ders-Bach 1971; Depucis & Berchias 1972; Loizou 1972; Tissari 1973).

Previous studies in our laboratories have demonstrated that acute 12% hypoxia in unanesthetized developing rats is accompanied by a decreased *in vivo* rate of brain tryptophan hydroxylation. This effect appeared to be somewhat less pronounced in the early postnatal period than later in development (Hedner *et al.* 1977a). A more severe hypoxia did only in a few cases result in a further decrease in enzyme activity (Hedner *et al.* 1978). When, however, the period of 12% hypoxia was prolonged to 6 h this resulted in a normalization of enzyme activity or in the neonatal rats, even an increased activity compared to controls (Hedner *et al.* 1977b).

Since the neonatal rats in this and certain other respects seem to react differently than older ani-

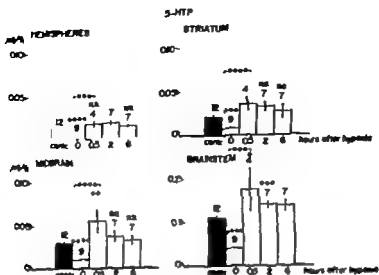


Fig. 3. 5-HTP levels in brain regions of 4-day-old rats during 30 min 6% hypoxia and recovery. Animals were injected subcutaneously with NSD 1015 (100 mg/kg, 30 min before sacrifice). Shown are means \pm S.E. Figures indicate the number of observations. $P < 0.05$ \leftrightarrow $P < 0.01$ \leftrightarrow $P < 0.005$ $P < 0.001$. When not specifically shown, significance is indicated versus controls.

rain was dissected out and immediately frozen on Al_2O_3 to the regional brain study the brain was re- and a "functional dissection" (Carlsson & al. 1973) was performed on an ice-cold glass plate. (1) striatum including corpus striatum and thalamus, (2) hemispheres including hippocampus, cerebellum ("midbrain") and (4) lower brain stem. As is removed and rejected. Most of the 5-HT cell bodies are found in part (4). The samples are stored at -70°C in a freezer in no case a slow 2 months. For the biochemical analyses, 4 pieces or 10 brain parts were weighed and pooled sample. The samples were homogenized in 25 ml tubes containing 10 ml perchloric acid, 0.2 ml 10% and 0.1 ml 5% $\text{Na}_2\text{S}_2\text{O}_8$. The extracts were purified roughly acidic cation exchange column (Dowex) and analysed for tryptophan, 5-HTP and 5-HT (Al. Magnusson 1970, Beilard et al. 1972, Atack & al. 1973).

Statistical analyses were performed using Student's t -test. Results are considered significant when $P < 0.05$.

$P < 0.05$ respectively). Control values were reached after 6 h. An increase in tryptophan levels ($P < 0.05$) compared to controls was however again observed 48 h after the anoxic period.

ii) In the regional brain study tryptophan levels tended to increase during hypoxia and subsequent recovery, however reaching significance only in the brain stem region 2 h after hypoxia (Table 1).

5-HTP levels

At the end of the 20 min anoxia there was a decrease in the amount of whole brain 5-HTP accumulated after NSD 1015 ($P < 0.05$) in the newborn animals (Fig. 2). After 30 min in the recovery phase 5-HTP accumulation increased approximately two-fold ($P < 0.001$). After this peak value a successive decline in the rate of 5-HTP accumulation was observed, reaching control values after 1 h and subnormal values ($P < 0.05$) 6 h after the nitrogen exposure. No changes compared to control animals were observed after 24 and 48 h.

iii) In all brain regions, 5-HTP accumulation after NSD 1015 similarly decreased during hypoxia (Fig. 3). An increase in 5-HTP accumulation was observed in all regions either 0.5 or 2 h after the 6% hypoxia. At 6 h after the hypoxia control levels were reached in all regions except for the brain stem

LTS

5-HTP levels

Exposure to nitrogen, whole brain tryptophan levels did not change in the 1-day-old animals (Fig. 1). In the recovery phase, there was a significant increase in tryptophan levels compared to control animals after 0.5 and 2 h ($P < 0.001$ and

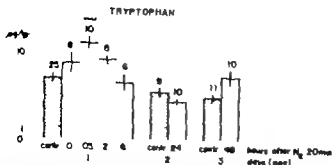


Fig. 1 Tryptophan levels in rat brain during nitrogen anoxia and recovery. Shown are means \pm S.E. Figures indicate the number of observations. $P < 0.001$, $P < 0.05$, n.s. = not significant.

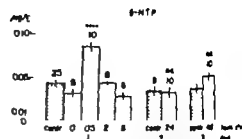


Fig. 2 5-HTP levels in rat brain during anoxia and recovery. Animals were injected subcutaneously with NSD 1015 (100 mg/kg, 30 min before sacrifice). Shown are means \pm S.E. Figures indicate the number of observations. $P < 0.001$, $P < 0.05$, n.s. = not significant.

imals to oxygen deprivation we have focussed our attention on the early postnatal period and designed this investigation so as to follow the 5-HT metabolism in the neonatal rat brain during asphyxia and the following recovery period.

METHODS

Pregnant Sprague-Dawley rats (Anticimex Stockholm) were obtained and housed in the department. The animals were kept in separate boxes under regulated dark-light conditions (light period 6 a.m. – 6 p.m.). The time of delivery was noted within 12 h.

The neonatal (4–36 h or 4 days old) rats were kept with their mothers the first day or days of life. Thereafter they were transferred to a 2-litre plastic cage. Experimental animals were rendered anoxic for 20 min with 100% N₂ or hypoxic for 30 min with 6% O₂–94% N₂. The gas mixture was passed through the plastic cage at a rate of about 4 l/min. Control animals were under similar conditions exposed to room air. The cages were kept on a preheated operating table (35–36°C). Room temperature was 22°C.

After anoxia or hypoxia (or corresponding period for

the control animals) some animals were killed immediately whereas the others were returned to their cages and allowed to breathe room air (see below). All rats were killed around 4 h after the onset of light.

Some of the neonatal rats (experimental and controls) were intended for brain analysis of injected 5-hydroxytryptophan (5-HTP) after inhibition of L-amino acid decarboxylase. These animals received subcutaneous injection of NSD 1015 (3-hydroxytryptophan HCl synthesized in this laboratory) (Lundberg) 100 mg/kg, dissolved in saline 10% they were killed. These animals as well as the analysed for brain 5-HT were killed immediately after oxygen deprivation (or corresponding period) or 0.5, 6, 24 or 48 h after exposure.

In a separate experiment aiming to study the pulse induced release of 5-HT during hypoxia, 16 rats were given a s.c. injection of 112.5 µg of depocetamide AB (Lyske Molindal) 400 mg/kg of the experiment and then rendered hypoxic (94% N₂). After 1 or 4 h in the hypoxic environment the animals were decapitated and the whole brain analysed for 5-HT. Zero control animals were kept with normal saline.

In all experiments the animals were decapitated

Table 1 Tryptophan levels in brain regions of 4 days old rats during 30 min of 6% hypoxia and room air

Shown are means \pm S.E. Figures within bracket indicate the number of observations. Significance is indicated by asterisks: $P < 0.05$, n.s. = not significant.

| | Hemispheres | Striatum | Midbrain | Brain stem |
|---------------------|------------------------------------|------------------------------------|-------------------------------------|-------------------------------------|
| Control | 5.7 \pm 0.44 (1) | 6.5 \pm 0.4 (1) | 6.5 \pm 0.39 (1) | 6.2 \pm 0.34 (1) |
| Hypoxia | 6.6 \pm 0.54 ⁿ (9) | 7.6 \pm 0.65 ⁿ (9) | 7.4 \pm 0.59 ⁿ (9) | 7.1 \pm 0.68 ⁿ (9) |
| 0.5 h after hypoxia | 6.2 \pm 0.88 ⁿ (7) | 7.3 \pm 0.93 ⁿ (7) | 7.5 \pm 0.70 ⁿ (16) | 7.4 \pm 0.66 ⁿ (15) |
| 2 h after hypoxia | 7.2 \pm 1.1 ⁿ (7) | 8.5 \pm 1.30 ⁿ (7) | 7.6 \pm 0.91 (7) | 7.9 \pm 0.89 ⁿ (7) |
| 6 h after hypoxia | 5.9 \pm 1.14 (7) | 6.9 \pm 1.18 ⁿ (7) | 7.5 \pm 0.85 ⁿ (7) | 7.0 \pm 0.79 ⁿ (7) |

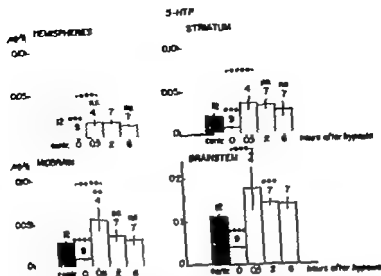


Fig. 3 5-HTP levels in brain regions of 4-day-old rats during 30 min 6% hypoxia and recovery. Animals were injected subcutaneously with NSD 1015 (0.1% 100 mg/kg, 30 min before sacrifice). Shown are means \pm S.E. Figures indicate the number of observations. $P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.005$ $^{****}P < 0.001$. When not specifically shown significance is indicated versus controls.

was detected out and immediately frozen on LiCl . In the regional brain study the brain was removed and "functional dissection" (Carlsson & al. 1973) was performed on an ice-cold glass plate. (1) striatum including corpus striatum and lenticulum, (2) hemispheres including hippocampus, neocortex ("isocortex") and (4) lower brain stem. Area was removed and rejected. Most of the 5-HT in the brain is found in part (4). The samples were stored at -70°C in freezer in no case than 2 months. For the biochemical analyses, 4 μmol or 10 μmol brain parts were weighed and pooled samples. The samples were homogenized in 25 μmol water, containing 10 μmol perchloric acid, 0.2 μmol 10% and 0.1 μmol 5% $\text{Na}_2\text{S}_2\text{O}_8$. The extracts were purified on a reversed phase cation exchange column (Dowex) and analysed for tryptophan, 5-HTP and 5-HT (A. Magnusson 1970, Bédard et al. 1972, Atack & al. 1973).

Statistical analyses are performed using Student's t -test. Values were considered significant when $P < 0.05$.

RESULTS

Whole brain levels

After exposure to nitrogen, whole brain tryptophan levels did not change in the 1-day-old animal (Fig. 1). In the recovery phase there was a significant increase in tryptophan levels compared to control animals after 0.5 and 2 h ($P < 0.001$ and

$P < 0.05$ respectively). Control values were reached after 6 h. An increase in tryptophan levels ($P < 0.05$) compared to controls was, however again observed 48 h after the anoxic period.

In the regional brain study tryptophan levels tended to decrease during hypoxia and subsequent recovery, however reaching significance only in the brain stem region 2 h after hypoxia (Table 1).

5-HTP levels

At the end of the 20 min anoxia there was a decrease in the amount of whole brain 5-HTP accumulated after NSD 1015 ($P < 0.05$) in the newborn animals (Fig. 2). After 30 min in the recovery phase 5-HTP accumulation increased approximately twofold ($P < 0.001$). After this peak value a successive decline in the rate of 5-HTP accumulation was observed reaching control values after 2 h and subnormal values ($P < 0.05$) 6 h after the nitrogen exposure. No changes compared to control animals were observed after 4 and 48 h.

In all brain regions 5-HTP accumulation after NSD 1015 similarly decreased during hypoxia (Fig. 3). An increase in 5-HTP accumulation was observed in all regions either 0.5 or 2 h after the 6% hypoxia. At 6 h after the hypoxia control levels were reached in all regions except for the brain stem

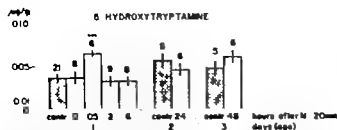


Fig. 4 5-Hydroxytryptamine levels in rat brain during nitrogen anoxia and recovery. Shown are means \pm S.E. Figures indicate the number of observations. $P < 0.005$ n.s. = not significant.

region where the increased 5-HTP accumulation persisted.

5-HT levels

Similarly to tryptophan and 5-HTP, 5-HT levels in the 1 day old animals had increased by about 75% 30 min after the nitrogen exposure ($P < 0.005$) (Fig. 4). No other changes in 5-HT levels compared to control animals were noted.

After blockade of the tryptophan hydroxylase with H22/54, no difference in the disappearance rate of 5-HT was noted between the hypoxia and control groups during 6% O_2 for 1 and 2 h in the 4 days old animals (Fig. 5).

DISCUSSION

Tryptophan hydroxylase is the first and probably rate limiting enzyme in serotonin formation in the brain (Costa & Neff 1970). In the rat brain the regional distribution of tryptophan hydroxylase activity parallels that of 5-HT (Schmidt & Sanders-Buch 1971; Deguchi & Barchas 1973). During development the increase in serotonin levels in the brain seems to parallel the increase in tryptophan hydroxylase activity (Bennet & Giarman 1965; Tisler 1973).

The second enzyme in the serotonin synthesis pathway, aromatic L-amino acid decarboxylase, can effectively be blocked by NSD 1015 (Carlsson et al. 1972). After blockade, the intermediate amino acid 5-HTP accumulates in a linear manner during the first 30 min. Thus, analyzing the amount of 5-HTP accumulated after NSD 1015 can be used as an accurate method to study serotonin synthesis (Carlsson et al. 1972).

Most of the oxygen consumed by the mammalian brain is used for energy production in the process of

oxidative phosphorylation. Apart from this fraction of the total oxygen requirement, hydroxylation reactions such as e.g. the synthesis of 5-HT and other monoamines, are also important.

Previous studies from this laboratory have shown that oxygen deprivation causes a decrease in the synthesis of the transmitters 5-HT, dopamine and noradrenaline (NA) in the neonatal rat (Hedner et al. 1977a, b, 1978). In these studies, 6–12% environmental hypoxia for 30 min inhibited 5-HTP accumulation in the brain. In the present study, oxygen deprivation reduced tryptophan hydroxylase activity by 30% compared to controls. All brain regions appeared to be similarly affected. No changes in levels of 5-HT or the precursor amino acid tryptophan were noted during oxygen deprivation.

The formation of 5-HTP from tryptophan requires the presence of molecular O_2 in the brain (Carlsson et al. 1977) as well as in the rat brain (Hedner et al. 1977a, b, 1978). This explains the decrease in 5-HTP accumulation during hypoxia in the present study. The depletion of the major 5-HT degrading enzyme, monoamine oxidase, may also explain the decrease in 5-HT levels during hypoxia.

The decrease in whole brain 5-HTP levels during anoxia was followed by a sharp rise after re-oxygenation. At this point in the recovery phase, there was also an increased availability of precursor tryptophan. Whole brain tryptophan levels were raised after hypoxia, and 5-HTP accumulation after NSD 1015 re-

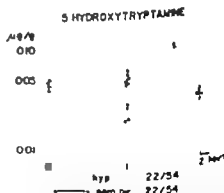


Fig. 5 5-Hydroxytryptamine disappearance rate in day-old rat whole brain during 6% hypoxia for 1 hour. H22/54 (α-propyltyrosineamide) 400 mg/kg injected 1 and 2 h before re-oxygenation, respectively.

after 2 h and even reached subnormal values.

The regional brain study for technical reasons based on 4 days old rats a similar course of for the levels of tryptophan and 5-HTP was seen in the whole brain. However in the brain regions where most of the 5-HT nerve cell bodies are located the increased 5-HTP levels per unit of the whole 6 h observation period after asphyxia. The explanation for this discrepancy between different brain regions can only be speculated. One possible explanation may be that the nerve cell bodies are more sensitive to hypoxia than other parts of the 5-HT neuron.

Tryptophan hydroxylase is not fully saturated with substrate tryptophan under normal conditions (Carlsson & Lindqvist 1978). The increase in 5-HT levels in the brain may in part reflect the decreased availability of tryptophan. On the other hand earlier studies have shown that tryptophan is a relatively ineffective substrate for increasing 5-HT levels in the neonatal rat brain (Bourgoignie et al. 1978). Other causes may also well be responsible for observed changes in 5-HTP accumulation after asphyxia. Such exogenous factors may for example be changes in enzyme activity or alterations in metabolism.

A consequence of the increased 5-HTP accumulation, brain 5-HT levels also increased during the immediate recovery from asphyxia, but were restored to normal later in the recovery period. No persistent effects on the brain serotonin levels were observed 48 h after the hypoxia.

Most of the changes caused by hypoxia on energy stores such as ATP and phosphocreatine in the brain seem to be readily reversible if the normal respiration and arterial circulation is restored. In order to understand the mechanisms responsible for the brain pathology produced by asphyxia, elucidation of early metabolic and structural changes occurring in the affected tissue is of considerable importance. As recent results from our study indicate that permanent alterations in serotonin metabolism can be caused by asphyxia (Hedner et al. 1979) this field of research attracts further investigation.

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Catecholamine metabolism in neonatal rat brain during asphyxia and recovery

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Infant rats were subjected to various degrees of asphyxia—1-day-old rats were exposed to 100% N₂ for 20 min and 4-day-old rats were exposed to 94% N₂-6% O₂ for 30 min. The activity of tyrosine hydroxylase, the first and rate limiting enzyme in the catecholamine (CA) synthesis, was studied *in vivo* in whole brain and in various brain parts by measuring the accumulation of dihydroxyphenylalanine (DOPA) after inhibition of aromatic L-amino acid decarboxylase with NSD 1015. Measurements of the endogenous levels of tyrosine, dopamine (DA) and noradrenaline (NA) were also made during asphyxia and recovery. During asphyxia there was a decrease in DOPA accumulation in the whole brain of the 1-day-old animals and in the striatum, midbrain and telencephalon regions of the 4-day-old rats. In the recovery phase both tyrosine and DOPA levels in the whole brain of the 1-day-old animals increased approximately 2-fold 30 min after asoxia. In the regional brain study a significant increase compared to controls was only noted in the striatum region. After 2 h, tyrosine and DOPA accumulation had reached normal or subnormal levels. In the 1-day-old rats there were no changes in whole brain DA or NA levels during asoxia and recovery except for an increase in DA levels noted after 6 h in the recovery phase. The disappearance rate of DA but not NA during hypoxia was retarded in the whole brain of the 4-day-old animals after H 44/68 (*α*-methyltyrosine), indicating a reduced nerve impulse activity in central dopaminergic but not noradrenergic nerves. The catecholamine neurotransmitters DA and NA are ascribed a lot of important functions in the mature brain. As hypoxia or asoxia cause profound changes in the metabolism of these transmitters during the neonatal period, this might be relevant as a factor underlying early childhood behavioural or neurological disorders.

Key words: Neonatal brain, asphyxia, dopamine, noradrenaline

Immature and developing nervous system of infant and neonatal animals exhibits a marked tolerance to anoxia and hypoxia. This seems to be a result of the low energy requirements of the neonatal brain (Hawthick et al. 1942; Jilek 1970; Lett 1976). In spite of these protective mechanisms, anoxia is followed by several cerebral metabolic responses (Vannucci & Duffy 1976). It has recently been reported that the catecholamine metabolism is affected already during relatively moderate hypoxia in the adult as well as in the neonatal rat brain (Davis & Carlsson 1973; Hedner et al. 1977a). The behavioural effects caused by the asphyxia, as suppression of locomotor activity and of the conditioned avoidance response, seem related to the decrease observed in

catecholamine metabolism (Brown et al. 1973; Brown & Engel 1973). These findings may explain why brain function during oxygen deprivation falls before there is any significant change in energy metabolism (Duffy et al. 1972). In previous studies from this laboratory we have shown that the activity of tyrosine hydroxylase, the first and rate limiting enzyme in the catecholamine synthesis pathway, decreases in the rat brain during 30 min of 12% hypoxia (Hedner et al. 1977a). The relative decrease appeared to be similar for the various ages studied. Additionally, only a slight further decrease in enzyme activity could be noted after decreasing the O₂ levels below 12% (Hedner et al. 1978). However, when extending the hypoxic period, enzyme activity tended to return to normal after 6 h in the

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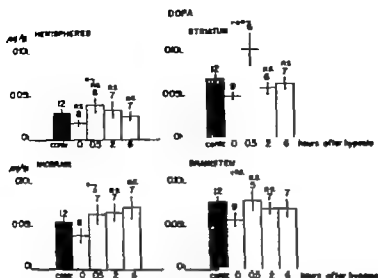


Fig. 3. DOPA levels in brain regions of 4-day-old rats during 30 min 6% hypoxia and recovery. Animals were injected subcutaneously with NSD 1015 100 mg/kg, 30 min before sacrifice. Shown are means \pm S.E. Figures indicate the number of observations. ** $P < 0.01$, * $P < 0.05$, s. = not significant. Where not specifically shown, significance is indicated versus controls.

brain) was quickly removed and dissected on an ice glass plate into the following parts: (1) striatum including corpus striatum and lenticular forniculation, (2) hemispheres including hippocampus, (3) diencephalon (midbrain) and (4) lower brain stem. Cerebellum was adenotomized and discarded. NA is the predominant catecholamine in parts (2) (3) and (4) and DA in part (1). Details of the dissection procedure, see Carlsson & van (1973). The samples were stored in freezer at -20°C in no case for more than 2 months. Four whole brains from the 1- and 4-day-old animals and 10 brain parts from 4-day-old animals were pooled for the biochemical analyses. The brain samples were homogenized in 10 ml perchloric acid containing 5 mg $\text{Na}_2\text{S}_2\text{O}_8$ and 20 JTA. The extracts were purified on a strong cation exchange column (Dowex 50-X-4) (Atack & Magnusson 1972) and analysed spectrofluorimetrically for tyrosine and DOPA or DA and NA according to previously described techniques (Waalhus & Udenfriend 1972, Atack & Magnusson 1970, Kehr 1972, Atack 1973). Statistical analyses were performed using Student's t -test. P -values larger than 0.05 were considered not significant.

TULTS

At the end of the

At the end of the nitrogen exposure the breathing of 1-day-old animals was found to be irregular and

infrequent. However the mortality during the 20 min exposure was very low, less than 5%. The 4-day-old animals exposed to 6% hypoxia were less affected and no mortality occurred during or after exposure.

Tyrosine levels

The influence of nitrogen exposure on the brain tyrosine levels in 1-day-old animals is presented in Fig. 1. The tyrosine levels did not change during the nitrogen exposure. A two-fold increase ($P < 0.001$) compared to control values was, however observed 0.5 h after exposure. Thereafter the tyrosine levels declined to normal after 2 h and after a further decline they were found to be below control values ($P < 0.005$). No difference was found between the experimental and control groups at 48 h after exposure. In the regional brain study performed in 4-day-old animals (Table 1) a significant decrease in tyrosine levels during hypoxia was only noted in the brain stem region ($P < 0.05$). During the recovery period tyrosine levels increased significantly in the hemispheres, striatum and the brain stem regions. The maximal increase was 27% in the hemispheres, 63% in the striatum and 63% in the brain stem region.

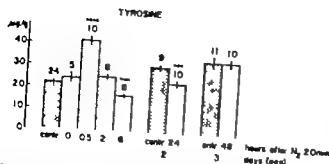


Fig 1 Tyrosine levels in 1-day-old rat brain during 20 min nitrogen anoxia and recovery. Shown are mean \pm S.E. Figures indicate the number of observations. $P < 0.001$ $P < 0.005$ n.s. = not significant.

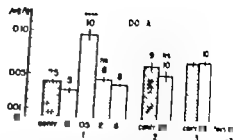


Fig 2 DOPA levels in 1-day-old rat brain during 20 min nitrogen anoxia and recovery. Animals were anaesthetized with NSD 1015 100 mg/kg. Wrote sacrifice. Shown are mean \pm S.E. Figures indicate number of observations. $P < 0.001$ $P < 0.005$ n.s. = not significant.

neonatal animals but not in the older age group (Hedner et al 1977b). These findings have prompted us further to investigate the alterations in catecholamine metabolism in the neonatal rat brain during anoxia or hypoxia and recovery.

METHODS

Pregnant Sprague-Dawley rats (Anticimex, Stockholm) were obtained at the 16th–18th day of gestation. The animals were housed in the department under regulated dark–light conditions (light period 6 a.m.–6 p.m.) and the time of birth was noted within 12 h. The neonatal animals were exposed to a hypoxic environment in a sealed 2-litre plastic cage. 1-day-old rats—100% N_2 for 20 min and 4-day-old rats—94% N_2 –6% O_2 for 30 min. 1 or 2 h after this exposure some neonates were returned to their dam allowed to breathe room air. All experiments were started around 4 h after onset of light. The nitrogen gas was passed through the cage via inlet and outlet holes at a rate of about 4 l/min. Control animals were kept in a similar box separated from the mother, exposed to room air. The

cages were kept on a preheated (35–36°C) water room temperature was 27°C.

At various intervals after the hypoxic exposure, experimental and control animals were analysed for content of either tyrosine and DOPA or DA and NA. 10 min before sacrifice all animals in which the whole brain or brain parts were analysed for tyrosine and DOPA, injected subcutaneously with NSD 1015 100 mg/kg. Hydroxybenzylhydrazine HCl synthesized in the laboratory by Dr Per Lundborg. The animals analysed for DA and NA did not receive any injection.

In a separate experiment 4-day-old animals, pretreated with H 44/68 (α -methyltyrosine) 50 mg/kg s.c. (zero controls). The H 44/68 treated animals were immediately exposed to 94% O_2 –6% N_2 or room air for 30 min. After this period the animals were decapitated and analysed for whole brain DA and NA.

The neonatal animals given NSD 1015 were killed immediately after 0.5, 6, 4 or 48 h after the 100% N_2 exposure or immediately after 0.5, 2 or 6 h after the 94% O_2 –6% N_2 exposure. After the decapitation the whole brain of the 1-day-old animals was dissected and immediately frozen on solid CO_2 . In the repeated study of the 4-day-old rats, the whole brain (without

Table 1 Tyrosine levels in brain regions of 4-day-old rats during 30 min of 6% hypoxia and recovery to room air

Shown are mean \pm S.E. Figures within brackets indicate the number of observations. Significance is indicated by asterisks. $P < 0.001$ $P < 0.005$ $P < 0.01$ $P < 0.05$ n.s. = not significant.

| | Hemispheres | Striatum | Midbrain | Brain stem |
|---------------------|-------------------------|------------------------|-------------------------|-------------------------|
| Control | 28.3 \pm 1.40 (10) | 33.4 \pm 1.81 (1) | 35.0 \pm 4.28 (8) | 33.7 \pm 1.66 (11) |
| Hypoxia | 26.7 \pm 1.9 (7) | 29.5 \pm 2.76 (9) | 31.9 \pm 2.33 (7) | 28.1 \pm 0.1 (9) |
| 0.5 h after hypoxia | 35.7 \pm 2.49* (7) | 49.0 \pm 5.34 (7) | 46.0 \pm 3.40* (6) | 44.0 \pm 3.94 (5) |
| 2 h after hypoxia | 33.2 \pm 3.71 (7) | 34.4 \pm 5.22 (6) | 49.0 \pm 9.33 (7) | 35.0 \pm 6.84 (7) |
| 6 h after hypoxia | 35.9 \pm 2.36 (7) | 41.3 \pm 3.76 (7) | 37.7 \pm 5.58* (7) | 46.5 \pm 6.58* (7) |

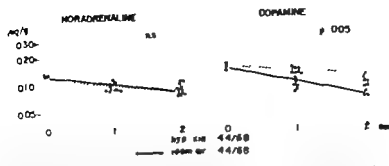


Fig. 6. Dopamine and norepinephrine disappearance curves in 4-day-old rat whole brain during 6% hypoxia for 1 and 2 h. H 44/68 (α -methyltyrosine) was injected 1 and 2 h before sacrifice, respectively.

accumulates in a linear manner. Measuring this can thus be used to study the *in vivo* activity of tyrosine hydroxylase (Carlsson et al. 1972). Cerebral cerebral metabolic responses occur during and after acute anoxia in newborn animals. Increased and potential energy stores such as glycogen, glucose, phosphocreatine and total adenine nucleotides decrease during anoxia, followed by a normalization or even an increase during the recovery phase (Berlet 1976). On the other hand, lactate increases in the brain during anoxia, a result of cerebral glycolysis as well as uptake from extracerebral sources. During recovery a successive decrease in brain lactate concentration takes place (Vannucci & Duffy 1976). Although the cerebral metabolic response to anoxia is extensive, brain function fails before there are any significant changes in ATP or phosphocreatine (Duffy et al. 1972). This phenomenon may in part be explained by the decreased synthesis of catecholamines, which has been found to be already at a moderate hypoxia or short anoxia (Carlsson & Carlsson 1973; Hedner et al. 1977). During 20 min of anoxia, the DOPA accumulation after NSD 1015 treatment was found to be decreased to about the same extent as has previously been demonstrated after a moderate hypoxia (2% O_2 during 30 min) (Hedner et al. 1977b). In the present study, 6% hypoxia significantly reduced brain DOPA accumulation after NSD 1015 in all the brain parts studied, except for the striatum region. Hence the changes in catecholamine caused by short term oxygen deprivation are evident in the neonatal rat brain at points where other metabolic patterns appear to be undisturbed.

After the anoxic period there was a marked increase in whole brain DOPA accumulation after NSD 1015, which may be the result of a variety of possible factors, such as increased oxygen tension, pH changes, arteriole shunting, alterations in energy metabolism or changes in neuronal transport or availability of precursors. The events in the various brain regions after hypoxia were similar but not identical. There was a more marked increase in tyrosine hydroxylase activity in the striatum region compared to the other areas 30 min after hypoxia. As for the whole brain the regional increase in tyrosine hydroxylase was short lasting and not detectable during further measurements 2 and 6 h after hypoxia. Hence these results indicate that the DA and NA neurons in the neonatal brain react similarly as the events occurring in a DA predominated area (striatum) almost parallel to those occurring in the NA predominated areas (hemispheres, mid-brain).

During the postanoxic phase the tyrosine levels in the whole brain as well as in the various brain regions increased and decreased in a way similar to that of DOPA. Although tyrosine hydroxylase is normally considered to be almost fully saturated with its precursor amino acid (Carlsson & Lindqvist 1978), increased levels of tyrosine might under certain conditions such as asphyxia be at least partly responsible for the increased levels of DOPA observed. As the enzyme tyrosine hydroxylase requires molecular oxygen for the synthesis of the new hydroxyl group of DOPA, the variations in DOPA accumulation observed during and after the anoxic period seem to indicate a decreased rate of catecholamine synthesis during anoxia and an increased rate in the immediate postanoxic phase.

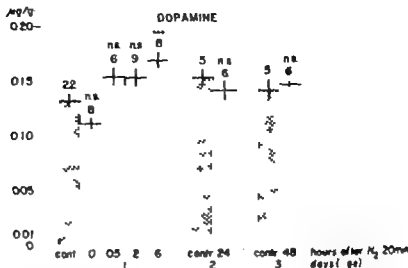


Fig. 4 Dopamine levels in 1-day-old rat brain during 20 min nitrogen anoxia and recovery. Shown are mean \pm S.E. Figures indicate the number of observations. $P < 0.005$ n.s. = not significant.

DOPA levels

The whole brain DOPA levels observed in the 1 day-old animals after treatment with NSD 1015 were significantly below control values ($P < 0.05$) immediately after 20 min in the 100% N₂ environment (Fig. 2). 30 min after the N₂ exposure the DOPA levels had increased markedly ($P < 0.001$) compared to the control group. From 2 h on after the exposure the DOPA levels had returned to normal.

In the various brain regions apart from the hemispheres in the 4-day-old rats (Fig. 3) 30 min of hypoxia induced a significant decrease in DOPA accumulation after NSD 1015. During the first 30 min of recovery from hypoxia DOPA levels increased significantly compared to control levels only in the striatum region. No changes compared to controls were noted 2 and 6 h after the 6% O₂-94% N₂ exposure for 30 min.

Dopamine and noradrenaline levels

No change in NA levels was found at any interval studied after N₂ exposure (Fig. 5). Neither did the DA levels change significantly during anoxia or recovery except at 6 h after the N₂ exposure where a slight increase compared to the control group was observed (Fig. 4).

Administration of H 41/68 caused a decline in DA and NA levels under control conditions. During the 2 h exposure to 6% O₂-94% N₂ the disappearance rate for brain DA was reduced ($P < 0.05$) (Fig. 6)

compared to controls. NA disappearance in brain did not differ between the control and experimental groups (Fig. 6).

DISCUSSION

The hydroxylation of tyrosine to DOPA is the rate limiting step in the catecholamine synthesis (Udenfriend 1966). Tyrosine hydroxylase has been detected in the rat brain at gestation and tyrosine hydroxylase is one of the first enzymes to appear in the catecholamine biosynthesis pathway (Coyle & Axelrod 1970). NSD 1015 is a potent inhibitor of the aromatic amino acid decarboxylase in the brain. It has been demonstrated that during the first 30 min after NSD 1015 administration the intermediate levels

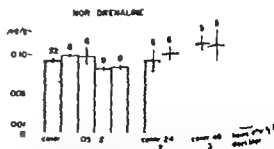


Fig. 5 Noradrenaline levels in 1-day-old rat brain during 20 min nitrogen anoxia and recovery. Shown are mean \pm S.E. Figures indicate the number of observations. $P < 0.005$ n.s. = not significant.

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Contrasting to these findings no major changes were observed in the endogenous whole brain levels of DA and NA. After synthesis inhibition the DA levels during hypoxia decreased more slowly than in the control situation. Thus the firing rate of DA neurons is inhibited during hypoxia in the neonatal brain. This inhibition of firing during hypoxia was not present for the NA neurons.

Although the degradation of CA was not investigated in the present study we can predict that during hypoxia there probably is a decrease in the degradation rate compensating for the decrease in synthesis rate. This is most certainly true for the NA neurons as neither the endogenous levels of the transmitter nor the firing rate of the neurons were altered during hypoxia. In fact studies in adult animals have demonstrated that monoamine oxidase but not catechol O-methyl-transferase is inhibited during oxygen deprivation (Davis & Carlsson 1973).

The changes occurring in the DA and NA neurons during and after a brief period of anoxia or hypoxia appeared to be relatively shortlasting and generally they could not be detected 24 and 48 h after the anoxic period.

A period of short oxygen deprivation has not been observed to induce any overt neuropathological changes in the cerebrum, cerebellum or brain stem of fetal and neonatal rats at the level of the light microscope (Vannucci & Duffy 1976). However when experimental animals have been subjected to prolonged partial asphyxia chronic neuropathological changes have been observed (Myers 1972; Vannucci & Duffy 1976). Similarly preliminary data from our laboratories indicate that prolonged oxygen deprivation results in permanent changes in CA metabolism in combination with certain behaviour disturbances (Hedner et al. 1979).

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Cerebral glucose consumption in the artificially ventilated rat: influence of nitrous oxide analgesia and of phenobarbital anesthesia

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INGVAR, A., ABDUL RAHMAN, A. & SIESJÖ, B. K. Local cerebral glucose consumption in the artificially ventilated rat: influence of nitrous oxide analgesia and of phenobarbital anesthesia. *Acta Physiol Scand* 1980, 109, 177-185. Received 15 Oct. 1979. ISSN 0001-6772. Laboratory for Experimental Brain Research, and Department of Neurosurgery, University of Lund, Sweden.

The objectives of the present study, which concerns local glucose consumption ($I-CMR_{gl}$) in the rat brain as measured with the ^{14}C -deoxyglucose technique of Sokoloff et al. (1977), were (1) to provide data for $I-CMR_{gl}$ in nitrous oxide analgesia and in phenobarbital anesthesia, allowing comparison with previous results on oxygen consumption, and (2) to test recent proposal that 70% N_2O markedly reduces $I-CMR_{gl}$ in (mainly) cortical structures. Under 70% N_2O , $I-CMR_{gl}$ in frontal and parietal cortex was close to 0.7 $\mu mol/g$ min. This value is in excellent agreement with previous values for cortical oxygen consumption. In phenobarbital anesthesia, $I-CMR_{gl}$ was lower than that expected from oxygen consumption, probably reflecting the fact that barbiturate anesthesia is accompanied by consumption of endogenous substrates. Experiments on adrenalectomized animals that were given local anesthesia and protected from external stimuli failed to demonstrate that 70% N_2O depresses $I-CMR_{gl}$. In fact, N_2O was found to increase $I-CMR_{gl}$ in many structures. It is concluded that if $I-CMR_{gl}$ is lower in ventilated animals than in spontaneously breathing, conscious controls, the depression is more likely to be due to the neuromuscular blockade.

Key words: Brain metabolism, local glucose consumption, nitrous oxide analgesia, phenobarbital anesthesia, neuromuscular blockade.

developed by Sokoloff et al. (1977) of the autoradiographic ^{14}C -deoxyglucose technique for measuring local cerebral glucose consumption in vivo ($I-CMR_{gl}$) has provided a new means of measuring cerebral metabolic rates in physiological and pathological situations. By virtue of its resolution, the method has the unique feature of allowing measurements of glucose consumption rates in distinct anatomical structures and functional units. In many novel metabolic information has been obtained on such diverse functions as those subserved by neural, auditory and olfactory systems (for details see Sokoloff et al. 1977; Sokoloff 1977) on the effect of α - and β -adrenoceptor antagonists (Savolainen et al. 1978) as well as on local metabolic rates accompanying focal and generalized epileptic seizures (Collins et al. 1976; Ingvar & Abdul-Rahman 1979), or those following

spreading cortical depression (Shiohara et al. 1979) and cerebral ischemia (Levy & Duffy 1977).

In any study of cerebral metabolic rate it becomes a problem to define an appropriate control state. Ideally such a state mimicks conditions prevailing in normal resting subjects (or experimental animals). Many pathophysiological conditions, particularly those involving changes in lung ventilation, blood pressure or body temperature require artificial ventilation. Furthermore immobilization is many times a prerequisite for appropriate sampling of tissue, cerebral venous blood, and CSF or for control of physiological parameters. In immobilized animals, the choice of anesthetic or analgesic drugs is crucial. In their review article Smith & Woolman (1972) conclude that, in man, 70% N_2O has small effect on CBF and only a mild depressant effect on CMR_{gl} . Experiments on dogs

phorylation rate of glucose and the amount of deoxy-6-phosphate formed which depends on the concentrations of the sugars and on their relative for the carrier mechanisms and for hexokinase as latter are known (see below) it is possible to calculate glucose utilization rates at steady state (which is phosphorylation rates) by measuring the time of the arterial plasma specific activities of the two and the deoxyglucose activity in tissue. For calculation of glucose utilization rates with the equation developed by Sokoloff et al. (1977) certain conditions should be fulfilled: (a) Plasma glucose concentrations should be constant during the experiment. (b) The tissue should occur in tracer concentrations. (c) Extracellular tissue compartment should be well mixed and all its parts should exchange directly with plasma. (d) Since measurements of ^{14}C -deoxyglucose activity in tissue are made by autoradiography it is not possible to distinguish between the free and phosphorylated forms. Hence a sufficient period should pass after injection of ^{14}C -deoxyglucose to allow most of it to disappear by phosphorylation or transformation of 1-CMR_{glc} is made from the following

$$1\text{-CMR}_{\text{glc}} = \frac{C_T(T)}{C_P} \cdot \frac{V_2 K_m}{V_1 K_m} \left[\left(\int_0^T \frac{C_T}{C_P} dt \right) - \frac{1}{k_1 + k_2} \left(\frac{C_T}{C_P} \right) e^{-(k_1 + k_2)T} \right]$$

where $1\text{-CMR}_{\text{glc}}$ is the glucose uptake rate per unit mass of tissue; C_T is the arterial plasma concentrations of glucose; C_P is the arterial plasma concentrations of glucose; T is the time of decapitation after injection; $C_T(T)$ is the amount of radioactivity in one of the tissue; k_1 and k_2 are the rate constants of mediated transport of deoxyglucose between plasma and tissue and between tissue and plasma, respectively; k_3 is the rate constant of phosphorylation of deoxyglucose; ϕ is the fraction of glucose that is further metabolized after phosphorylation; K_m , V_2 , K_m , V_1 are the Michaelis-Menten rate and maximal phosphorylation rates for deoxy and deoxyglucose respectively applying the equation T was 45 min and the constants given by Sokoloff et al. (1977).

Measure of 1-CMR_{glc} procedures

^{14}C -deoxyglucose (37 mCi mmol, New England) in 0.5 ml of Krebs solution was infused over 4 of 30 min at infusion pump 15 times 100 μl (including blank) were taken during the 45 min infusion period. The samples were centrifuged and plasma separated and frozen at -80°C for later measurement of ^{14}C -activity and glucose concentration.

45 min the animal was decapitated hereafter the brain was removed and frozen in isopentane chilled to -80°C . Plasma glucose concentrations were determined enzymatically with the hexokinase method (see Folberg et al. 1977). The ^{14}C -deoxyglucose activity was determined by preparing an aliquot of plasma into scintillation vial containing 1 ml of mixture of Soluene³ and isopropanol (1:1 v/v). To the vial were then added 7.5 ml of mixture of Instagel³ and 0.1 N HCl (9:1 v/v). The samples were counted in Nuclear Chicago liquid scintillation counter. The efficiency was calculated with the method of external standard ratio. The accuracy of this method was evaluated by adding an internal standard (^{14}C -hexadecane, Amersham) to the vials before recounting.

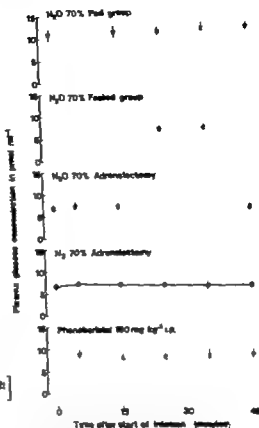


Fig. 1. The plasma glucose concentrations just before the start of deoxyglucose infusion and during the 45 min period of the experiments. The values are means \pm S.E.

terminated by preparing an aliquot of plasma into scintillation vial containing 1 ml of mixture of Soluene³ and isopropanol (1:1 v/v). To the vial were then added 7.5 ml of mixture of Instagel³ and 0.1 N HCl (9:1 v/v). The samples were counted in Nuclear Chicago liquid scintillation counter. The efficiency was calculated with the method of external standard ratio. The accuracy of this method was evaluated by adding an internal standard (^{14}C -hexadecane, Amersham) to the vials before recounting.

Determination of ^{14}C -activity in the tissue by autoradiography was done as described previously (Abdul-Rahman et al. 1979). When recalibrating the standards it was found that the values previously assigned to them were in error by about 5%. This was due to the fact that the volume of internal standard used was sufficient to induce some quenching. Accordingly the CBF values given in that article (Abdul-Rahman et al. 1979) are about 5–10% too low.

RESULTS

The physiological parameters in the 5 experimental groups are given in Table 1. All animals had rectal

(Theye & Michenfelder 1968) and rats (Carlsson et al 1976) have given similar results indicating that 70% N_2O may provide an adequate control state in immobilized animals.

So far extensive control data for $1-CMR_{ad}$ have been published only for unanesthetized and partially restrained rats and monkeys and for rats subjected to superficial thiopental anesthesia (Sokoloff et al 1977 Sokoloff 1978 Kennedy et al 1978). Recently Sakurada, Shinohara, Kennedy & Sokoloff (manuscript in preparation) compared $1-CMR_{ad}$ values obtained in spontaneously breathing rats that were either partially restrained by a plaster cast or freely moving to those measured in immobilized animals ventilated on 70% N_2O . Since their results indicate that 70% N_2O depresses $1-CMR_{ad}$ in cortical structures by up to 35% the authors question the assumption that values so obtained approximate those expected in the resting conscious state.

The present experiments were undertaken to critically assess $1-CMR_{ad}$ values obtained in ventilated rats maintained on 70% N_2O . The following questions were posed: (1) Are the $1-CMR_{ad}$ values measured in reasonable agreement with those expected from CMR_{O_2} values previously obtained in immobilized rats? In order to obtain data for this comparison $1-CMR_{ad}$ was measured in animals maintained on 70% N_2O as well as in those given phenobarbital in a dose of 150 mg/kg. These situations provide both "normal" and markedly reduced metabolic rates. Furthermore since many experiments are conducted in fasted rats $1-CMR_{ad}$ under 70% N_2O was assessed both in fed rats and in those fasted for 24 h. (2) Does 70% N_2O depress glucose utilization in the brain? In order to answer this question $1-CMR_{ad}$ was measured in animals maintained either on 70% N_2O -30% O_2 or on 70% N_2 -30% O_2 . In all of these animals pain and stressful reactions were minimized by adrenalectomy and local anesthesia.

METHODS

Operative techniques and experimental groups

The experiments were performed on male Wistar rats of a SPF strain weighing 370-420 g. In the majority of the animals (see below) anesthesia was induced with 3% halothane. When unresponsive the animal was tracheotomized and immobilized with an i.v. injection of tubocurarine chloride (0.5 mg/kg). Ventilation was continued with a gas mixture of 70% N_2O and 0.7% halothane

in oxygen for the duration of the operation. Its arteries were cannulated, one for recording blood pressure and the other for obtaining blood samples for determination of blood gases, pH, glucose, and ^{14}C -deoxyglucose activity. One femoral vein was cannulated to allow infusion of drugs and ^{14}C -deoxyglucose. Body temperature, as measured in the rectum, was kept close to 37°C by external heating. Upon completion of operative procedures the halothane supply was discontinued and heparin was given i.v. in a dose of 100 units/kg.

The following 5 groups of animals were used in the study.

1 *Fasted*-70% N_2O . 6 animals were fasted for 24 hours prior to the experiment. Following the operation they were maintained on 70% N_2O .

2 *Fed*-70% N_2O . In this and in the following groups each comprising 4 animals, the animals had free access to pellet food until the day of operation. This group otherwise respects similar to group 1.

3 *Adrenalectomy-local anesthesia*-70% N_2O . In this group the adrenal glands were removed prior to operation. Local anesthesia (about 1 ml of 1% lidocaine per animal) was infiltrated at suture sites. The rats' ears were plugged with cotton wool, they were given a sterile ointment and then almost completely covered with cotton wool (see Carlsson et al 1977). Ventilation was then continued on 70% N_2O .

4 *Adrenalectomy-local anesthesia*-70% N_2O . This group was identical to the previous one except for that nitrous oxide supply was discontinued before ^{14}C -deoxyglucose injection.

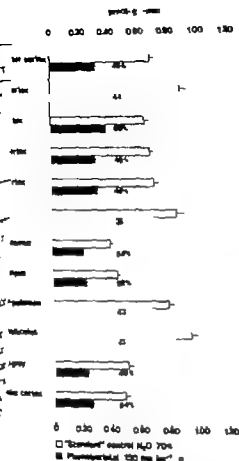
5 *Phenobarbital* 150 mg/kg. This group was different from the others in that anesthesia was induced with phenobarbital (150 mg/kg i.p.). However the rats were ventilated with 70% N_2O and 0.7% halothane during operation at the end of which ventilation was continued on 70% N_2 and 30% O_2 .

After the completion of operative procedures the animals were allowed a steady state period of 30 min. The determination of $1-CMR_{ad}$ was begun (see below) in group 4. During this time it was controlled that P_{aO_2} was in the range 35-40 mmHg and that P_{aCO_2} was 35-40 mmHg.

Determination of $1-CMR_{ad}$ -theoretical background

The ^{14}C -deoxyglucose technique is based on the following considerations: Deoxyglucose is transported from the blood to cerebral intracellular fluids by the same mechanisms as glucose and like glucose is phosphorylated by hexokinase. However the metabolism of deoxyglucose is impeded by the fact that it is a poor substrate for glucose-6-phosphatase (E.C.5.3.1.9) and for glucose-6-phosphate dehydrogenase (E.C.1.1.1.49). Since the deoxyglucose-6-phosphate (E.C.3.1.3.9) activity is low in brain tissue the deoxyglucose is essentially trapped in the tissue as deoxyglucose-6-phosphate. The amount trapped at any given time is proportional to the rate of phosphorylation by hexokinase minus the rate of dephosphorylation by glucose-6-phosphatase.

A quantitative relationship exists between



The influence of phenobarbital anesthesia on local cerebral glucose consumption (cmr_{gl}) in the rat as compared with the 70% N_2O group. The numbers denote 1- CMR_{gl} phenobarbital anesthesia as percent of the standard.

were pooled for the comparison to results obtained by Sakurada et al. in areas with high metabolic rates (inferior colliculus and cerebral cortex) present values were lower than those of Sakurada et al. the differences being about 30% in inferior colliculus and 10-20% in cortical areas. However the relative proportion of 1- CMR_{gl} between structures was very similar. In some other structures (particularly thalamus, hypothalamus, neocortex and cerebellar cortex) to some extent the values in the two series were in close agreement. In still others, a comparison could not be made since the densitometer in the present study precluded measurements in very small structures.

Table 3 Comparison of local glucose consumption (CMR_{gl}) in frontal and parietal cortex and oxygen consumption (CMR_{O_2}) in animals ventilated on 70% N_2O or given 150 mg/kg of phenobarbital

| Anesthesia | CMR_{O_2} measured | CMR_{gl} calculated | CMR_{gl} found |
|--------------------------|------------------------------------|-------------------------------------|--------------------------------|
| 70% N_2O | 4.0* | 0.70 | 0.70 |
| Phenobarbital 150 mg/kg | 2.7* | 0.47 | 0.32 |

Bernstam et al. (1979)

Nilsson & Siesjö (1975), with one aberrant value excluded (see discussion in article quoted).

2. Local CMR_{gl} phenobarbital anesthesia

In view of the fact that the phenobarbital-anesthetized animals were paralyzed and artificially ventilated it would seem most relevant to compare the values obtained to those of the "standard" group. Fig. 2 shows the absolute values obtained in the two groups and lists the percentage reduction in CMR_{gl} induced by the barbiturate in each structure studied. In most of the structures the reduction in 1- CMR_{gl} was to about 50% of control but there were exceptions. Thus, the reduction in CMR_{gl} was somewhat less pronounced in the visual cortex and somewhat more pronounced in inferior colliculus. In the thalamus, the reduction was excessive (to a third of control).

3. A comparison between glucose and oxygen consumption rates

Previously CMR_{O_2} has been measured in animals maintained on 70% N_2O (e.g. Bernstam et al. 1979) and in those given phenobarbital 150 mg/kg (Nilsson & Siesjö 1975) experimental conditions being similar to the present ones. In these studies we used a modification of the Kety & Schmidt (1948) technique with sampling of cerebral venous blood from the caudal portion of the superior sagittal sinus. Assuming that this sampling site provides CMR_{O_2} values representative of mainly frontal and parietal cortex and that the CMR_{gl} values measured come close to the weighted averages for these structures we lumped together the values for these two cortical areas for comparisons. Furthermore we assumed that 95% of the glucose consumed is oxidized the remainder appearing as lactate (and pyruvate) in the venous effluent (see Hawkins et al. 1973; Norberg & Siesjö 1974). The resulting calcu-

Table 1 *Physiological parameters in experimental groups*The values are means \pm S.E. MABP = mean arterial blood pressure

| Experimental groups | Temp (°C) | MABP (mmHg) | P (mmHg) | P _{CO₂} (mmHg) | pH |
|--|-----------------|-------------|-------------|------------------------------------|------|
| N ₂ O 70% fed group (n=4) | 37.0 \pm 0.05 | 143 \pm 5 | 115 \pm 2 | 39.1 \pm 0.4 | 7.37 |
| N ₂ O 70% fasted group (n=6) | 37.1 \pm 0.1 | 135 \pm 6 | 113 \pm 2 | 38.8 \pm 0.3 | 7.35 |
| N ₂ O 70% adrenalectomy + local anesthesia (n=4) | 37.1 \pm 0.2 | 121 \pm 7 | 104 \pm 3 | 37.9 \pm 1 | 7.30 |
| N ₂ O 70% adrenalectomy + local anesthesia + N ₂ O (n=4) | 37.3 \pm 0.1 | 118 \pm 6 | 110 \pm 3 | 37.4 \pm 0.8 | 7.29 |
| Phenobarbital 150 mg/kg (n=4) | 37.4 \pm 0.1 | 108 \pm 5 | 117 \pm 3 | 39.0 \pm 1 | 7.28 |

temperatures close to 37°C. Pa_O values close to or above 100 mmHg. Pa_{CO} values around 40 mmHg and pH values close to 7.40. There was a tendency for blood pressure to decrease in the adrenalectomized animals ($P<0.05$) and in those anesthetized with phenobarbital blood pressure was clearly decreased below "control" (70% N₂O, $P<0.01$).

Fig. 1 shows the plasma glucose concentrations just before the start of deoxyglucose infusion and during the 45 min period of the expt. In all groups except that comprising the fed animals under 70% N₂O plasma glucose concentrations were in the range 5–8 μ mol/g. As required (see Methods)

plasma glucose concentrations were constant throughout the period of the expt. with little variation between individual animals.

Local CMR_D in nitrous oxide analysis

Table 2 lists 1-CMR_D values obtained in the 7 of animals ventilated on 70% N₂O and compared them to those measured by Sakurada et al. (in preparation) under similar conditions (N₂ and neuromuscular blockade). Local CMR_D values differ between fed and starved animals except cerebellar cortex which had a slightly higher value in the starved group ($P<0.05$). In view of the similarity in results between fed and starved

Table 2 *Local glucose consumption (1-CMR_D) in animals ventilated on 70% N₂O with or without fast of 18–20 h, as compared to data obtained by Sakurada et al. (in preparation) under similar conditions (see text)*The values are means \pm S.E.

| Structures | Fasted n=6 | Fed n=4 | Combined n=10 | Sakurada et al. n=9 |
|--|-----------------|-----------------|------------------|-------------------------------|
| A Superficial cerebral structure cortex | | | | |
| Sensorimotor | 0.68 \pm 0.04 | 0.69 \pm 0.04 | 0.69 \pm 0.03 | 0.80 \pm 0.03 |
| Auditory | 0.9 \pm 0.07 | 0.85 \pm 0.04 | 0.89 \pm 0.04 | 1.10 \pm 0.06 |
| Visual | 0.69 \pm 0.03 | 0.60 \pm 0.02 | 0.65 \pm 0.03 | 0.78 \pm 0.04 |
| Parietal | 0.69 \pm 0.04 | 0.69 \pm 0.0 | 0.69 \pm 0.02 | 0.79 \pm 0.02 ^{ns} |
| Frontal | 0.67 \pm 0.03 | 0.76 \pm 0.03 | 0.71 \pm 0.01 | 0.87 \pm 0.02 ^{ns} |
| B Deep cerebral structure | | | | |
| Hypothalamus | 0.45 \pm 0.03 | 0.37 \pm 0.01 | 0.41 \pm 0.01 | 0.43 \pm 0.04 |
| Caudate-putamen | 0.86 \pm 0.05 | 0.71 \pm 0.03 | 0.80 \pm 0.04 | 0.87 \pm 0.06 |
| C Mid-brain and Pons | | | | |
| Inferior Colliculus | 1.00 \pm 0.06 | 0.87 \pm 0.04 | 0.95 \pm 0.04 | 1.30 \pm 0.11 |
| Pontine gray | 0.55 \pm 0.04 | 0.49 \pm 0.03 | 0.52 \pm 0.03 | 0.51 \pm 0.05 |
| D Cerebellum | | | | |
| Cerebellar cortex | 0.55 \pm 0.03 | 0.4 \pm 0.01 | 0.50 \pm 0.03 | 0.4 \pm 0.03 |

$P<0.05$ $P<0.01$

DISCUSSION

These results are highly pertinent to the problem of obtaining proper control conditions for studies of physiological and pathophysiological processes in the brain. When discussing the results it must be recalled, though, that such conditions vary from one study to another. Clearly for studies of many physiological events it must be possible to compare results with those obtained in anesthetized and spontaneously breathing animals. It would seem that for these the control conditions used by Sakurada et al. (in preparation) see Sokoloff et al. (1977) are the appropriate ones. It has not been excluded, though, that values so obtained are influenced by the stress of partial restraint of infusion and/or blood sampling in freely moving animals (see below).

As stated in the introduction, other studies require immobilization and some forms of anesthesia, e.g. that provided by 70% N_2O . The question has remained whether or not such immobilization markedly alters brain metabolism and blood flow. In the following, we will discuss the implications of the present results for these general problems.

Validity of $I-CMR_{glc}$ value in animals anesthetized on 70% N_2O

Because of the difficulty of estimating a weighted average CMR_{glc} value for any given cerebral structure referring $I-CMR_{glc}$ and CMR_{O_2} values to a defined structure. However, since similar values were obtained if repeated density readings were taken from different parts of the frontal and parietal cortices (data not shown) and since values for frontal parietal and sensorimotor cortex were similar and since the sagittal sinus probably drains mainly cortical structures, we conclude that the present $I-CMR_{glc}$ values quantitatively correspond to previous measurements of CMR_{O_2} . Since the values are close to those measured with a kinetic ^{14}C -glucose technique (Bergstrom et al. 1976) there should be no doubt about the quantitative validity of the values obtained. True in cerebral cortical structures and in the inferior colliculus, our CMR_{glc} values were moderately but significantly lower than those measured by Sakurada, Shimohama, Kennedy & Sokoloff (in preparation). However, the differences were small enough to be explained by differences

in metabolic characteristics between the rat strains used (Sprague-Dawley versus Wistar) or by unrecognized differences in experimental conditions. Such differences may explain why Sakurada et al. obtained local CBF values exceeding 2.2 ml/g/min in all cortical areas except the visual cortex. To our knowledge, flow rates of this magnitude have not been recorded previously.

One further point should be emphasized in view of the fact that about 20% of the brain oxygen consumption is accounted for by ketone body oxidation after a 24-hour fast (Ruderman et al. 1974); it may seem paradoxical that glucose consumption rates were similar in fed and fasted animals. However, since an increased proportion of glucose is metabolized to lactate (and pyruvate) in fasted animals and since arteriovenous differences in glucose concentration remain virtually unchanged (Hawkins et al. 1971; Ruderman et al. 1974) the present results are not surprising.

Validity of $I-CMR_{glc}$ values in animals under phenobarbital anesthesia

The primary purpose of performing measurements on phenobarbital-anesthetized animals was to allow a comparison between CMR_{glc} and CMR_{O_2} . At first sight, it was surprising that a good correspondence was obtained in animals on 70% N_2O but not in those anesthetized with phenobarbital. In barbiturate-anesthetized animals, the calculated CMR_{glc} value ($0.47 \mu\text{mol/g/min}$) even if somewhat too high (see discussion of CBF technique by Bernitsman et al. 1979) clearly exceeds that measured with the autoradiographic technique in the appropriate cortical areas. We tentatively conclude that the difference is due to the fact that actual metabolic rate (i.e. oxygen consumption) exceeds glucose utilization. This can be explained by the fact that barbiturates retard glycolysis at the phosphofructokinase step leading at least initially to mobilization of endogenous carbohydrate and amino acid substrates for oxidation (Chapman et al. 1978). If so, barbiturate anesthesia provides an example of conditions in which glucose and oxygen consumption rates are partly dissociated.

Previously the influence of thiopental on $I-CMR_{glc}$ has been assessed (Sokoloff et al. 1977). Local CMR_{glc} was reduced by 40–50% in areas with high metabolic rate, particularly of cerebral cortical origin. Since thiopental was administered in doses that just extinguished the corneal reflex, the values

Table 4 Comparison of $I\text{CMR}_{gl}$ in animals that were adrenalectomized and given local anesthesia and ventilated on either 70% N_2O or 70% N_2

The values are means \pm S.E.

| Structures | 70% N_2O | 70% N_2 |
|---|--------------------------|------------------|
| A Superficial cerebral structures cortex | | |
| Sensorimotor | 0.73 ± 0.04 | 0.64 ± 0.04 |
| Auditory | 0.97 ± 0.05 | 0.72 ± 0.03 |
| Visual | 0.81 ± 0.05 | 0.63 ± 0.02 |
| Parietal | 0.69 ± 0.07 | 0.63 ± 0.04 |
| Frontal | 0.81 ± 0.04 | 0.64 ± 0.04 |
| B Deep cerebral structures | | |
| Thalamus | 1.05 ± 0.05 | 0.67 ± 0.03 |
| Hypothalamus | 0.48 ± 0.02 | 0.41 ± 0.01 |
| Hippocampus | 0.73 ± 0.01 | 0.55 ± 0.03 |
| Caudate putamen | 1.07 ± 0.05 | 0.75 ± 0.04 |
| C Mid-brain and Pons | | |
| Inferior Colliculus | 0.81 ± 0.06 | 0.66 ± 0.06 |
| Pontine gray | 0.55 ± 0.01 | 0.44 ± 0.04 |
| D Cerebellum | | |
| Cerebellum cortex | 0.5 ± 0.02 | 0.49 ± 0.04 |

$P < 0.05$

$P < 0.01$

$P < 0.001$

lutions (Table 3) showed an excellent agreement between expected and measured cortical CMR_{gl} in animals on 70% N_2O . However in phenobarbital anesthetized animals the autoradiographically measured CMR_{gl} values were lower than those expected from the CMR_{gl} measurements (see Discussion).

4 The influence of nitrous oxide analgesia

As stated a previous study showed no significant difference in CMR_{gl} between animals ventilated on 70% N_2O and 70% N_2 (Carlsson et al 1976). In these stress pain and discomfort were minimized by removal of the adrenal glands by local anesthesia and by measures instituted to reduce external stimuli. Using an identical preparation we measured glucose consumption rates with and without 70% N_2O (Table 4). Unexpectedly the results showed that 70% N_2O increased $I\text{-CMR}_{gl}$ in many of the structures studied. Thus although there were no or only small differences in sensorimotor and parietal cortex, hypothalamus, inferior colliculus, pontine gray and cerebellar cortex, other structures (frontal, auditory and visual cortex, thalamus, hippocampus and caudate putamen) showed signifi-

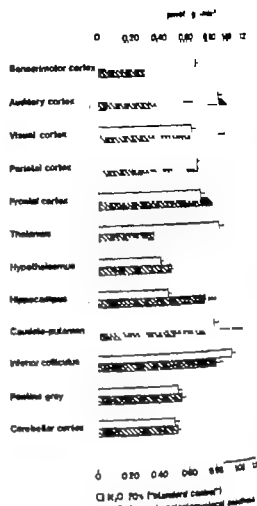


Fig. 3 Local CMR_{gl} in adrenalectomized and anesthetized animals ventilated on 70% N_2O (open bars) as compared to standard control animals on 70% N_2O (hatched bars). The values are means \pm S.E. in $\mu\text{mol/g/min}$. $P < 0.001$.

cantly increased CMR_{gl} with 70% N_2O . These results seem to exclude the possibility that N_2O reduces metabolic rates in the brain (see Discussion).

Clearly as Fig. 3 shows a complex emerges when the results obtained on adrenalectomized nitrous oxide animal (Table 4) are compared to those obtained in the standard control animal (Table 3). Thus in spite of the fact that both groups were artificially ventilated on 70% N_2O and that they were adrenalectomized, injected with anesthetic and protected against external stimuli, the animals had higher $I\text{-CMR}_{gl}$ values in frontal and parietal cortex, thalamus, hippocampus and caudate putamen and a lower value in inferior colliculus. We conclude that one or more of these procedures influence local glucose consumption.

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breathing and artificially ventilated animals. It could reflect a reduction in cerebral metabolism due to the neuromuscular blockade, and associated reduction in afferent stimuli. Somewhat possible that partial restraint and exposure of freely moving animals induce a stress effect, i.e. increase in $1-CMR_{gl}$ which is reduced when ventilated animals are ventilated on 70% N_2O . Evidence that some stress could be present is published by Böhrer et al. (1978) who have shown that gentle handling of rats for 30 s caused the adrenaline concentration to rise to about 100% of basal values.

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Relationship between plasma pH and pancreatic HCO₃⁻ secretion at different intravenous secretin infusion rates

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RÆDER, N., MO, A., AUNE, S. & MATHISEN, Ø. Relationship between plasma pH and pancreatic HCO₃⁻ secretion at different intravenous secretin infusion rates. *Acta Physiol Scand* 1980, 109: 187-191. Received 19 Oct. 1979. ISSN 0001-6772. Institute for Experimental Medical Research, University of Oslo, and Surgical Dept. 2, Ullevål Hospital, Oslo.

The relationship between pancreatic HCO₃⁻ secretion and plasma pH during acute systemic acid-base changes was investigated in 6 anesthetized, artificially ventilated pigs (20-25 kg) at 2 different, secretin infusion rates. At 0.45 C.U./kg b wt. h⁻¹ secretin infusion and plasma pH 7.40±0.01 pancreatic HCO₃⁻ secretion averaged 61±12 µmol/min. Stepwise lowering of plasma pH through i.v. infusion of HCl and CO₂ administration to inspired air proportionately reduced secretion rate: estimated zero HCO₃⁻ secretion occurring at plasma pH 7.01. Subsequent i.v. secretin infusion at 2.70 C.U./kg b wt. h⁻¹ increased HCO₃⁻ secretion to 249±42 µmol/min at plasma pH 7.33±0.04; stepwise lowering of plasma pH proportionately reduced HCO₃⁻ secretion to estimated zero at plasma pH 6.71. A reduction of plasma pH by 0.1 pH unit reduced HCO₃⁻ secretion during low and high rate of secretin infusion by 10±3 µmol/min and 35±8 µmol/min, respectively. Secretin infusion rate did not affect pancreatic chloride excretion. These findings support the view that secretin increases HCO₃⁻ secretion, and hence proton transport to the interstitial fluid, by augmenting the proton motive force developed by HCO₃⁻ secreting cells.

Key words: Acid-base hypothesis, pig, proton motive force, proton pump

pancreatic HCO₃⁻ secretion implies equimolar transport of protons to the interstitial fluid by secreting cells (Davies 1949). It has recently been demonstrated that plasma pH determines the rate of pancreatic HCO₃⁻ secretion: during intravenous secretin infusion stepwise lowering of plasma pH proportionately reduces HCO₃⁻ secretion; secretion is abolished at plasma pH 6.90 (et al. 1979). Secretin is a major stimulant of pancreatic HCO₃⁻ secretion but it is not clear how the relationship between plasma pH and pancreatic HCO₃⁻ secretion varies with secretin infusion rate. One possibility is that secretin increases HCO₃⁻ secretion as it lowers the plasma pH value at which HCO₃⁻ secretion is abolished by acidosis. This would harmonize with the view that secretin increases HCO₃⁻ secretion through raising the proton motive force developed by HCO₃⁻ secreting cells. The other pos-

sibility would be that HCO₃⁻ secretion would increase upon augmenting secretin infusion rate at any plasma pH exceeding 6.90; at plasma pH <6.90 the mechanism responsible for HCO₃⁻ secretion might be refractory to secretin stimulation. This would be in accordance with the view that acidosis reduces HCO₃⁻ secretion through inhibiting the metabolism in HCO₃⁻ secreting cells, near complete metabolic inhibition occurring at plasma pH 6.90.

The purpose of the present study was to distinguish between these alternatives. The relationship between plasma pH and pancreatic HCO₃⁻ secretion was studied in 6 anesthetized pigs during acute systemic acid-base disturbances at two different rates of i.v. secretin infusion. We found that increasing i.v. secretin infusion from 0.45 to 2.70 C.U./kg b wt. h⁻¹ increased both the rate of pancreatic HCO₃⁻ secretion and lowered the plasma pH limit at which HCO₃⁻ secretion is abolished by

plasma pH (correlation coefficient, $r=0.98$). HCO_3^- secretion ultimately ceased at a secretin infusion rate of 0.45 C.U./kg . Lowering of plasma pH by 0.1 unit reduced pancreatic HCO_3^- output by an average of $2.70 \mu\text{mol/min}$, estimated zero secretion occurred at plasma pH 7.01 . The inhibition of HCO_3^- secretion at low plasma pH was reversible as shown by the return of HCO_3^- secretion upon normalization of plasma pH through withdrawal of CO_2 from inspired air/ O_2 mixture and further back-titration of plasma pH with i.v. NaHCO_3 infusion. At a secretin infusion rate was increased to $2.70 \text{ g b.wt. h}^{-1}$ pancreatic HCO_3^- secretion rose to $42 \mu\text{mol/min}$ at plasma pH 7.33 ± 0.04 and was significantly higher ($P < 0.05$) than during $0.45 \text{ g b.wt. h}^{-1}$ secretin infusion rate at all plasma pH values between 7.60 and 8.76 . During step-wise lowering of plasma pH at $2.70 \text{ C.U./kg b.wt. h}^{-1}$ secretin infusion rate, pancreatic HCO_3^- secretion fell in proportion to changes in plasma pH (Fig. 1); a reduction in plasma pH by 0.1 unit under these circumstances reduced pancreatic output by $35 \pm 8 \mu\text{mol/min}$, which is significantly more than the reductions in HCO_3^- secretion observed during lowering of plasma pH at $0.45 \text{ g b.wt. h}^{-1}$ secretin infusion rate. Estimated zero secretion occurred at plasma pH 6.71 during $2.70 \text{ C.U./kg b.wt. h}^{-1}$ secretin infusion rate. Thus, in addition to augmenting pancreatic HCO_3^- output, increase in secretin infusion rate shifted the plasma pH limit at which HCO_3^- secretion was abolished. Under these conditions, zero secretion was $14 \pm 1 \mu\text{mol/min}$ at plasma pH 6.99 ± 0.01 during low secretin infusion rate and $39 \pm 0.01 \mu\text{mol/min}$ at plasma pH 7.33 ± 0.04 during high secretin infusion rate. The difference is not likely that secretin had no certain effect on chloride transport under these circumstances (Table 1).

DISCUSSION

The present study demonstrates a linear relationship between plasma pH and pancreatic HCO_3^- secretion at both high and low rates of i.v. infusion of secretin. Increasing secretin infusion rate from 0.45 to $2.70 \text{ C.U./kg b.wt. h}^{-1}$ raised pancreatic HCO_3^- secretion and lowered the plasma pH limit at which HCO_3^- secretion was abolished by acidosis from pH 7.01 to pH 6.71 . Furthermore the reduction in

HCO_3^- secretion elicited by a decimal unit reduction of plasma pH was greater during high than low rate of i.v. secretin infusion.

Recently we suggested that a proton pump in the interstitial cell membrane of HCO_3^- secreting cells may be responsible for proton transport to the interstitial fluid (Røder et al. 1979). Changes in plasma pH proportionately alters HCO_3^- secretion rate while changes in pancreatic juice pH elicited by altering arterial P_{CO_2} are without effect on secretion rate; thus, HCO_3^- secretion is affected by pH changes only at the interstitial cell border of HCO_3^- secreting cells (Røder et al. 1979). The reason for this asymmetric sensitivity of HCO_3^- secreting cells to external pH is not clear. One explanation could be that reductions in interstitial fluid pH could increase back leakage of protons across the interstitial cell membrane and thus reduce net transport of protons from HCO_3^- secreting cells. Alternatively reductions in interstitial fluid pH could increase the electrochemical proton potential of interstitial fluid and curtail the proton potential difference between HCO_3^- secreting cells and interstitial fluid. Accordingly secretion would cease when the proton potential difference over the interstitial cell membrane was zero. Finally interstitial fluid proton concentration could inhibit metabolism in HCO_3^- secreting cells; changes in metabolic rate and hence energy supply to the proton transport mechanism, could cause proportionality between the rate of proton transport and plasma pH.

The effects of secretin observed in the present study harmonize with the view that secretin increases the proton motive force generated by HCO_3^- secreting cells. An increase in proton motive force would both augment proton transport rate and sustain proton transport at higher interstitial fluid proton concentrations. We found greater increments in HCO_3^- secretion per decimal pH unit increase in plasma pH at high secretin infusion rate than would indicate that the proton motive force increases more with plasma pH under these conditions.

Augmenting secretin infusion rate restored HCO_3^- secretion at plasma pH < 7.01 . This indicates that acidosis does not impair proton transport through reducing metabolism in pancreatic cells. If metabolism were suppressed to the point that it could no longer sustain proton transport at plasma pH 7.01 increasing secretin infusion rate from 0.45 to $2.70 \text{ C.U./kg b.wt. h}^{-1}$ would not be expected to yield any secretory response from the pancreas at

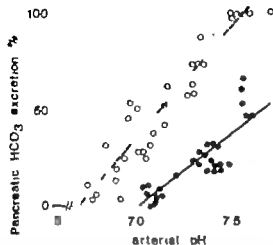


Fig. 1 Relationship between pancreatic HCO_3^- excretion and plasma pH during acute acid-base changes in anesthetized secretin infused pigs. Plasma pH was varied by infusion of HCl intravenously and addition of CO_2 to the respired air/ O_2 mixture. 100% = maximal secretory rate obtained in each experiment. \bullet = 0.45 C.U. secretin/kg b.w.t. h⁻¹; \circ = 2.70 C.U. secretin/kg b.w.t. h⁻¹. Oblique lines represent calculated linear regression for relationship between HCO_3^- excretion and arterial pH at the two different secretin infusion rates. Each animal served as its own control. Number of expts. 6.

acidosis from pH 7.01 to pH 6.71. A reduction in plasma pH by 0.1 pH unit reduced HCO_3^- secretion more at high i.v. secretin infusion rate than at low infusion rate. These findings favour the view that secretin may increase pancreatic HCO_3^- secretion through an effect on the force driving protons from HCO_3^- secreting cells to the interstitial fluid.

METHODS

The experiments were carried out on 6 young pigs (20–25 kg) during general pentothal anesthesia (40 mg/kg b.w.t.) additional pentothal was given i.v. as required during the expts. Anesthesia was supplemented by periodic administration of pancuronium bromide (Pavulon). The animals were artificially ventilated, an endo-tracheal tube ensured free airways. All animals were killed upon completion of the expts.

Blood pressure was monitored by a Statham transducer (P23Gb) via a polyethylene catheter inserted into one carotid. Vagal nerves were left intact. Both jugular veins were cannulated, one for secretin infusion, the other for electrolyte infusions.

The abdomen was opened by a midline incision. For timed collection of pancreatic juice, the main pancreatic duct was cannulated by a soft polyethylene catheter through a duodenotomy incision. The gallbladder and urinary bladder were drained by indwelling catheters. The

common bile duct and pylorus were occluded with clamps. During the operation 500 ml 0.9% NaCl solution was infused i.v. Following surgery the abdomen was closed with clips. The body temperature of the animal was kept constant by an electric pillow.

Secretin (from the Karolinska Institute, Sweden) was infused i.v. at a steady rate of 0.45 C.U./kg b.w.t. h⁻¹ during the initial half of the expts. subsequently a further infusion of 30 C.U. secretin was injected i.v. as a bolus. The infusion continued at 2.70 C.U./kg b.w.t. h⁻¹.

Upon reaching steady state after continuous infusion, paired 4 min samples of pancreatic juice were obtained. Arterial blood specimens were drawn in the middle of each collecting period for electrolyte and immediate pH and P_{CO_2} determination. Timed collection of pancreatic juice (300 µl per pig) was sampled on capillary tubes for amylase and P_{CO_2} determination.

Plasma pH was lowered from control by continuous administration of HCl i.v. up to a total of 40–60 mmol, dissolved in 300–500 ml water. Further lowering of pH was achieved through stepwise increase of P_{CO_2} in a dioxygen mixture.

At each step during steady state 4 min collections of pancreatic juice and blood specimens were drawn for analysis. Arterial pH was subsequently re-normalized to P_{CO_2} in inspired air mixture and the titration of plasma pH with 100–160 mmol NaHCO₃ 900–1000 ml water with 5 mmol HCl added. The procedure for lowering plasma pH in steps was repeated at secretin infusion rate of 2.70 C.U./kg b.w.t. h⁻¹. Collections of pancreatic juice and blood were made at each step.

Pancreatic juice, plasma pH and P_{CO_2} were measured in Instrumentation Laboratories Blood Gas Analyzer (Lexington, Mass., U.S.A.) and plasma HCO_3^- concentration was calculated according to Henderson-Hasselbalch equation. Pancreatic juice HCO_3^- concentration was estimated as the difference between the sum of Na⁺ concentrations and Cl^- concentration in pancreas. Na⁺ and K⁺ concentrations in plasma and pancreas were measured by an Instrumentation Laboratories flame photometer. Plasma and pancreatic juice determinations were made with a CM 10 chloride titration (Copenhagen, Denmark).

Statistical methods

Wilcoxon's rank test for unpaired measurements and Whitney test) was used to evaluate differences between observations (Snedecor 1967). Linear regression was calculated using the method of least squares fit.

RESULTS

At control plasma pH 7.40 ± 0.01 , pancreatic secretion averaged $78 \pm 9 \mu\text{mol/min}$ during infusion of secretin at 0.45 C.U./kg b.w.t. h⁻¹. Secretion of pancreatic juice decreased with further lowering of plasma pH through i.v. infusion of HCl and addition of CO_2 to the inspired air/ O_2 mixture. The reduction in HCO_3^- secretion was proportional to the

| HCO_3^- ($\mu\text{mol/min}$) | Cl^- ($\mu\text{mol/min}$) |
|---|--|
| 78 | 13 |
| ± 9 | ± 1 |
| 249 | 18 |
| ± 42 | ± 3 |

lowever the positive relationship of plasma concentration to HCO_3^- secretion which is reported (Case et al 1970 Pak et al 1966 1972) may not be causative as different relationships pertain between HCO_3^- secretion and HCO_3^- concentration at normal and high a contrast, there is an unequivocal relationship between plasma pH and HCO_3^- secretion under conditions (Rader et al 1979) Plasma pH may be the relevant ionic determinant of rate of HCO_3^- secretion during acid-base m. Furthermore trebling of pancreatic juice concentration as during high P_{O_2} is without effect on the rate of secretion (Rader et al) the proposed HCO_3^- -ATPase mechanism suggests that an increase in proton supply at the cell border enhances proton transport observations therefore, argue against the validity of holding luminal membrane bound HCO_3^- as responsible for pancreatic HCO_3^- secretion

results of the present study therefore may be interpreted within the framework of pancreatic HCO_3^- secretion being due to a pancreatic proton pump orientated towards the interstitial and responding quantitatively to secretin stimulation by increasing the proton motive force

This study was supported by the Norwegian Research Council for Science and the Humanities Professor Carl Semb Fund for Medical and Surgical Research, Doctor Alexander Mathie's Legacy and Ullevaal Hospital Gastroenterological Research Fund. The experiments were carried out at the Institute of Experimental Medical Research, University of Oslo. The skilled assistance of Miss Ann Kristin Andreassen, Miss Inger Bjerkedal, Mrs Patricia Cooper, Mrs Anna Croucher, Mrs Ellen Dahl, Mrs Nina Eriksson, Mr Erik Faust Olsen, Mr Bjørn Kristiansen, Mr Severin Larnard B.Sc, Mrs Onthe Lærum, Mr Ove Moen and Mrs Åshild Salvesen is gratefully acknowledged.

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Table 1 Pancreatic juice composition and secretion at different intravenous secretin infusion rates

| Secretin infusion rate | Arterial concentrations | | | Pancreatic juice concentrations | | | | | |
|------------------------|-------------------------|---------------------------|------------------------------|---------------------------------|---------------------------|------------------------------|----------------|---------------|----------------|
| | pH | Pco ₂ (kPa) | HCO ₃ (mmol/l) | pH | Pco ₂ (kPa) | HCO ₃ (mmol/l) | Na (mmol/l) | K (mmol/l) | Cl (mmol/l) |
| 0.45 C.U./kg b.wt. h | 7.40 ±0.01 | 5.37 ±0.26 | 22.9 ±0.4 | 8.14 ±0.02 | 4.79 ±0.20 | 133 ±5 | 154 ±1 | 44 ±0.2 | 1 |
| 2.70 C.U./kg b.wt. h | 7.33 ±0.04 | 6.48 ±0.03 | 24.8 ±2.1 | 8.16 ±0.02 | 5.14 ±0.39 | 144 ±4 | 151 ±1 | 49 ±0.4 | 5 |

Data presented are mean values \pm S.E. from 6 expts. on anesthetized young pigs (20–25 kg) each animal served as control.

plasma pH < 7.01. Also the immediate return of HCO₃ secretion and proton transport upon normalization of plasma pH after acidosis argues against extensive interference with metabolism in HCO₃ secreting cells.

Secretin could increase HCO₃ secretion through reducing back-leakage of protons from the interstitial fluid into HCO₃ secreting cells. This could be the case if secretin reduced interstitial cell membrane proton permeability. However the concept of proton back-leakage as regulator of net proton transport during acute acid-base changes implies that proton back-leakage would increase with increasing interstitial fluid proton concentration. The inhibition of HCO₃ secretion elicited by decimal unit reduction of plasma pH increased in the present study when secretion infusion rate was raised. This would indicate more rather than less proton back-leakage under these circumstances. Therefore the possibility that secretin may increase HCO₃ secretion through reducing proton permeability of the interstitial cell membrane of HCO₃ secreting cells is viewed as unlikely.

So far the results of our study have been discussed with reference to the proton pump hypothesis. However other possibilities should be considered. It has been proposed that pancreatic proton flux is secondary to transcellular Na transport (Swanson & Solomon 1972). According to this hypothesis two Na-H ion exchange mechanisms are present in pancreatic cells—one in the interstitial cell membrane, the other in the luminal cell membrane of HCO₃ secreting cells. The former pump would be driven by the electrochemical potential for Na movement into cells; the latter pump would be linked directly to metabolism (Swanson & Solomon 1975). The energy available for proton secretion from pancreatic cells would be proportional to the logarithm of the ratio between Na concentra-

tions outside and inside secretory cells, for one to one ion exchange ratio under these conditions the ion exchange would be independent of cell membrane potential (Swanson & Solomon 1975).

To account for how secretin may increase the force driving protons to the interstitial fluid by Na-H ion exchange mechanism one might postulate that secretin would lower intracellular concentrations in HCO₃ secreting cells, thus increase the potential energy available for Na-H exchange at the interstitial cell membrane. However it has been reported that cell Na concentration increases with the onset of HCO₃ secretion (Scratcherd & Case 1973). Alternatively an increase in driving force would result if the Na-H exchange ratio was increased by secretin, i.e. Na ions moving into the cell per proton as secretin infusion rate was augmented would either establish an Na carousel and Na-H ATPase at the interstitial cell border or to increased transcellular transport of NaCl. In the present study we could find no evidence of an increasing pancreatic transport of NaCl.

It has also been proposed that pancreatic HCO₃ secretion is mediated by HCO₃-ATPase located in the luminal cell membrane. According to this scheme protons would be actively transported by HCO₃-ATPase from duct lumen across the luminal cell membrane into cells where they would be buffered by HCO₃ ions diffusing into the cells from interstitial fluid. The ensuing carbonic acid would dehydrate to CO₂ which would diffuse into the lumen where it would rehydrate, ionize and release HCO₃ ions for excretion as well as carbonic OH ions with formation of HCO₃ ions that would recycle (Schultz et al. 1971). Secretion would be proportional to HCO₃ delivery into secretory cells—i.e. proportional to plasma HCO₃ concentration.

Constant flow vs constant pressure-perfusion studies of pulmonary vasoactive responses

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BJERTNÆS L. & HAUGE, A. Constant flow- vs. constant pressure-perfusion for studies of pulmonary vasoactive responses. *Acta Physiol Scand* 1980, 109: 193-200. Received 23 Oct. 1979. ISSN 0001-6772. Institute of Physiology, University of Oslo, Norway.

We have compared the pulmonary vascular responses to standardized hypoxic vasoconstrictor stimulus ($F_{iO_2}=0.02$) obtained during 1) constant volume inflow with pulmonary arterial pressure as the dependent variable, and 2) constant inflow pressure, with flow as the dependent variable. Isolated rat lungs were perfused at different baseline transvascular pressures. The experimental arrangement allowed changes between the two types of perfusion. Hypoxia at constant pressure perfusion gave a higher percentage rise in pulmonary vascular resistance (PVR) at all pressure levels. This advantage was however more than offset by (a) the finding that a) vascular closure (total or partial) often occurred, particularly below arterial pressure of 3 kPa, making detection of graded responses impossible and b) the control situation was rarely regulated. Responses obtained during constant flow were less reduced by elevations in baseline transvascular pressure and the control situation was rapidly and completely regained. The observation that hypoxic vascular closure may occur in the pulmonary vascular bed supports the hypothesis that high altitude edema is caused by precapillary occlusion of a major part of the vascular bed, thereby subjecting still perfused regions to very high pressures and flow.

Key words: Long vessels, vascular smooth muscle, arteriolar wall stress, transmural pressure, hypoxia, critical closure.

We have explored the relative merits of constant flow and constant pressure perfusion systems for the assessment of a vasoconstrictor response in the pulmonary circulation. As the standardized stimulus for vasoconstriction, we used a hypoxic ventilation gas mixture ($F_{iO_2}=0.02$). In order to quantitate the pulmonary vascular response to acute alveolar hypoxia and the effect on the response caused by general anesthesia we have previously used a constant flow perfusion technique (Hauge 1968, Bjertnæs 1977). A rise in vascular resistance caused by hypoxic vasoconstriction was measured as a rise in pulmonary arterial pressure. This commonly used technique for studies of pulmonary vasoconstrictor-stimuli recently been criticized (Benumof & Wahren 1975). It was pointed out that the induced rise in intravascular pressure will attenuate the response making the test-system rapidly less sensitive as the constrictor-stimulus is increased. A constant pressure system with flow as the dependent

variable should consequently be a better technique for evaluation of pulmonary vascular responses.

The degree of attenuation of the vasoconstrictor response during constant flow perfusion will depend on several factors such as the radius of the vessels involved and the level of initial circumferential smooth muscle stretch. Gore (1977) has investigated the relationship between magnitude of active constriction and initial stress in the arterioles of the frog mesentery but this relationship is not known for the small pulmonary arteries involved in the response to hypoxia.

Because there is no general agreement on the advantages of constant pressure as compared with constant flow perfusion we decided to compare the vasoconstrictor responses obtained at the two modes of perfusion at different intravascular pressure levels. In addition we have discussed the significance of our findings in relation to high altitude pulmonary edema and the phenomenon of critical closure of vessels.

constant flow vs constant pressure-perfusion studies of pulmonary vasoactive responses

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Key words: Lung vessels, vascular smooth muscle, arteriolar wall stress, transmural pressure, hypoxia, critical closure.

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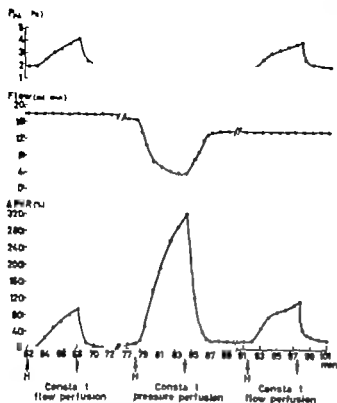


Fig. 1 Recordings of pulmonary arterial pressure (P_{pa}) blood flow and calculated changes in pulmonary vascular resistance (ΔPVR). Three 6 min periods of ventilation hypoxia are indicated by arrows (H). The first and the third response to hypoxia is obtained during constant flow, the middle during constant pressure perfusion. Base line (control) arterial pressure was the same. A slight flow reduction also reflected as a small increase in PVR occurred on changing from constant flow to constant pressure perfusion.

The hypoxic stimulus resulted in the largest response expressed as ΔPVR , during constant pressure perfusion but baseline flow and PVR were not regained.

METHODS

Operative technique

Rats of the Wistar strain weighing between 240 and 260 g were exposed to diethyl ether until they became unconscious. Pentobarbital 3–4 mg/100 g was then injected intraperitoneally.

A tracheostomy was performed and continuous positive pressure ventilation started. The chest was opened through a median sternal approach. The trachea, the lungs and the main vessels were dissected free from adjacent tissue. The pericardium was opened. The esophagus was ligated just above the diaphragm and cut. A loose ligature was tied around the inferior caval vein above the diaphragm and a second one around the pulmonary artery, aorta and the superior caval vein just above heart level. Half a ml containing 40 IU pure powdered heparin (No. 6) in isotonic saline was slowly injected into the left ventricle. The ligature around the inferior caval vein was

tightened and the ventilation stopped. The preparation was then removed from the chest, the steel cannula fitted with two extra inlets, one for monitoring and one for constant pressure perfusion inserted into the pulmonary artery and the ligatures removed. Similarly a larger stainless steel cannula was inserted through the left ventricle into the left atrium. Both cannulae and the silicone rubber tubing of the perfusion system were provided with luer lock connections for rapid coupling, thereby reducing the pulmonary blood flow. The time which elapsed between arrest of the animal's own circulation and perfusion was between 5 and 13 min (mean 10 min).

The lung preparation with cannulae was air humidified in a perspex chamber surrounded by a stated water jacket where it hung freely against inflow cannula.

Perfusion

The vascular bed was perfused via the arterial constant volume inflow or constant inflow pressure experiment was always started with constant flow using a roller pump (LKB Sæderlind). The effluent was drained into a thermostated perspex chamber. From a bottom outlet the perfusate entered the pulmonary artery to the lungs, and then it was through the left atrial cannula to the top inlet of the reservoir. This was covered by a piece of Teflon, sealed only by a hole for the venous tubing. The pulmonary arterial pressure and flow was measured. Statham P 23Db pressure transducer and flow transducer connected to a Sanborn polygraph. The venous pressure was also measured by collecting effluent in a graduated cylinder for standardized periods of time. In few experiments left atrial pressure elevations were carried out by leading the effluent blood directly to the desired connection in a vertical ladder of perspex and silicon rubber tubing. Stepwise elevation of pressure was obtained by closing the appropriate tube connections for the time periods required.

Constant inflow pressure perfusion was achieved by pumping the blood from the venous reservoir in a ladder of perspex and silicon rubber tubing from the arterial side. Inflow pressure could be set at a level by clamping the rubber tube connection at the desired level above the lungs. From this level the blood flowed into the arterial cannula. Any excess forced by the pump and not accepted by the pulmonary bed under the prevailing pressure was drained back into the blood reservoir. In the drainage pathway was clamped. Constant inflow pressure during a vasoconstrictor response was obtained by manual reduction in pump output. The purpose of this arrangement was to maintain the pulse amplitude also at the lowest flow levels. During the experiment we found that by this arrangement the mean inflow pressure could be kept constant within ± 0.13 kPa (1 mmHg) flow reduction. Mean and undamped pulmonary pressure was measured simultaneously.

Intillation

After one or two gentle intillations of the lung preparation with a constant inflow pressure perfusion was started using a Statham

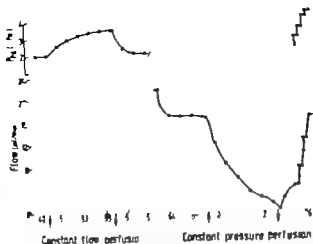


Fig. 2 Recordings of pulmonary arterial pressure (P_{pa}) and flow. Vasoconstrictor responses to hypoxia (between arrows) were evoked, first during constant flow perfusion, then during constant pressure perfusion, starting from the same baseline (control) arterial pressure. A spontaneous flow reduction occurred when perfusion was changed from one of constant flow to one of constant pressure. During the subsequent hypoxic period flow fell to zero. Control flow level was only regained after stepwise increases in arterial pressure to twice the initial level. Abscissa is time after start of lung perfusion.

pump (C. H. Palmer Ltd). Tidal volume was set at 2 ml (constant in each exp.), giving peak wave of about 1 kPa at start of perfusion and

The ventilation frequency was fixed at 80 r/min. End expiratory pressure was kept constant at 0.2 kPa by means of a water seal. Tracheal air was recorded by differential pressure transducer (270, Hewlett Packard), connected to the recorder. The lungs were ventilated with gas consisting of 21% oxygen, 5% carbon dioxide, nitrogen. Airway hypoxia was created by ventilating lungs with gas mixture containing 2% O_2 , 5% carbon dioxide and 93% nitrogen. The present experimental model are modifications of those by Hauge (1964, b).

was

0 ml blood from 3 to 5 anesthetized donor rats, noted by heart puncture. Each 10 ml of blood of 10 IU of heparin (Novo). Air was evacuated from perfusion system (tubings and connections) by Krebs-Ringer solution through a. Just prior to long perfusion, the perfusate blood (which had red for about 1 h in closed perspex bottle at 37°C) was substituted for the Krebs-Ringer solution. pH was controlled by using Radiometer acidimeter (PH4711).

LTS

ventilation hypoxia elicited a vasoconstrictor response a few second after the entrance of the $100\% O_2$ mixture ($F_{iO_2} = 0.02$) into the alveoli

Fig. 1 gives examples of responses obtained during constant flow and constant pressure perfusion in one pair of lungs. The period of hypoxia was 6 min each time and the vasoconstriction was measured either as a rise in pulmonary arterial pressure or as a reduction in flow. When the response is expressed as a change in pulmonary vascular resistance (ΔPVR) the standardized vasoconstrictor stimulus used in this example caused a response which was 3.5 times larger during constant pressure perfusion than during perfusion with constant flow.

Fig. 2 gives another example in which the hypoxic period was 6 min during both modes of perfusion. First, a response to hypoxia obtained during constant flow perfusion was detected as a reversible rise in pulmonary arterial pressure. When a constant pressure perfusion was established, flow was transiently reduced before the correct inflow pressure was obtained. This purely passive mechanical type of event resulted in lasting PVR increase as reflected in the new lower flow. When the hypoxic stimulus was introduced an unstable situation was created. The vasoconstriction resulted in a zero flow condition in spite of unaltered pulmonary arterial pressure. When the hypoxic stimulus was withdrawn only a modest increase in flow occurred. In order to regain flow it was necessary to increase inflow pressure. Thus it appeared

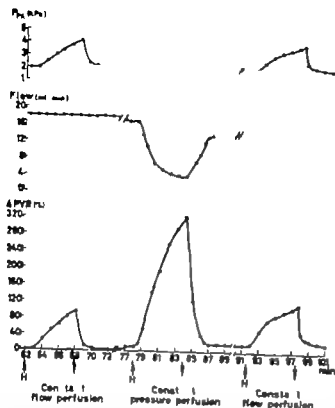


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tightened and the ventilation stopped. The preparation was then removed from the chest, a steel cannula fitted with two extra lumen, one for monitoring and one for constant pressure perfusion inserted into the pulmonary artery and the left ventricle. Similarly a larger stainless steel cannula was inserted through the left ventricle into the aorta. Both cannulae and the silicone rubber tubing of the perfusion system were provided with interlocking flanges for rapid coupling, thereby reducing the possibility of pulmonary blood flow. The time which elapsed between arrest of the animal's own circulation and perfusion was between 5 and 13 min (mean 8 min).

The lung preparation with cannulae was put in a humidified perspex chamber surrounded by a static water-jacket where it hung freely except inflow cannula.

Perfusion

The vascular bed was perfused via the arterial constant volume inflow or constant inflow perfusion experiment was always started with constant flow using a roller pump (LKB Sweden). The effluent was drained into a thermostated perspex reservoir. From a bottom outlet the perfusate was pumped through the pulmonary artery to the lungs, and then out through the left atrial cannula to the top outlet of the reservoir. This was covered by a piece of 'parafilm' sealed only by a hole for the venous tubing. Pulmonary arterial pressure and flow was measured with a Statham P 23Db pressure transducer and a flow transducer connected to a Sashimi polygraph was also measured by collecting effluent into a graduated cylinder for standardized periods. In a few experiments left atrial pressure was also carried out by leading the effluent blood stream to the desired connection in a vertical ladder of perspex and silicon rubber tubing. Stepwise elevation of pressure was obtained by closing the appropriate tube connections for the time periods required.

Constant inflow pressure perfusion was achieved by pumping the blood from the venous reservoir in a ladder of perspex and silicon rubber tubing above the arterial side. Inflow pressure could be set at a desired level by clamping the rubber tube connections at the desired level above the lungs. From this level the blood flowed into the arterial cannula. Any excess blood was not accepted by the pulmonary vascular bed under the prevailing pressure but drained back into the blood reservoir. In 2 out of 12 experiments a drainage pathway was clamped. Constant mean pressure during a vasoconstrictor response was obtained by manual reductions in pump output. The purpose of this arrangement was to maintain the pulse pressure amplitude also at the lowest flow-levels during flow reductions. We found that by this arrangement the mean pressure could be kept constant within ± 0.13 kPa during flow reductions. Mean and undamped pulmonary pressure was measured simultaneously.

Initial inflation

After one or two gentle inflations of the lungs, pressure inflation was started using a Statham

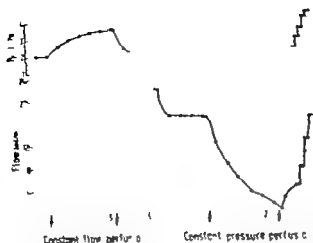


Fig. 2. Recordings of pulmonary arterial pressure (P_{pa}) and flow. Vasoconstrictor responses to hypoxia (between arrows) are evolved first during constant flow perfusion, then during constant pressure perfusion, starting from the same baseline (control) arterial pressure. A spontaneous flow reduction occurred when perfusion was changed from one of constant flow to one of constant pressure. During the subsequent hypoxic period flow fell to zero. Control flow level was only regained after stepwise increases in arterial pressure to twice the initial level. Abscissa is time after start of lung perfusion.

on pump (C. F. Palmer Ltd). Tidal volume was 1 and 2 ml (constant in each split), giving peak pressure of about 1 kPa at start of perfusion and flow. The ventilation frequency was fixed at 80 per min. End expiratory pressure was kept constant at 0.2 kPa by means of water seal. Tracheal pressure is recorded by differential pressure transducer (Model 770, Hewlett-Packard), connected to the recorder. The lungs were ventilated with gas consisting of 21% oxygen, 5% carbon dioxide and 74% nitrogen. Airway hypoxia was created by ventilating the lungs with gas mixture consisting of 5% carbon dioxide and 95% nitrogen. The preparation and experimental model are modifications of those used by Hauge (1968a, b).

Flow
40 ml blood from 3 to 5 anesthetized donor rats, removed by heart puncture. Each 10 ml of blood was 10 U of heparin (Novo). Air was evacuated by perfusion system (tubings and connections) by using Krebs-Ringer solution through it. Just prior to start of lung perfusion, the perfusate blood (which had stored for about 1 h in closed perspex bottles at 7°C) was substituted for the Krebs-Ringer solution. pH was controlled by using Radiometer acid-analyser (PHM71).

RESULTS

Exhalation hypoxia elicited vasoconstriction a few seconds after the entrance of the hypoxic gas mixture ($P_{O_2} = 0$ kPa) into the alveoli.

Fig. 1 gives examples of responses obtained during constant flow and constant pressure perfusion in one pair of lungs. The period of hypoxia was 6 min each time, and the vasoconstriction was measured either as a rise in pulmonary arterial pressure or as a reduction in flow. When the response is expressed as a change in pulmonary vascular resistance (ΔPVR) the standardized vasoconstrictor stimulus used in this example caused a response which was 3.5 times larger during constant pressure perfusion than during perfusion with constant flow.

Fig. 2 gives another example in which the hypoxic period was 6 min during both modes of perfusion. First, a response to hypoxia obtained during constant flow perfusion was detected as a reversible rise in pulmonary arterial pressure. When a constant pressure perfusion was established flow was transiently reduced before the correct inflow pressure was obtained. This purely passive, mechanical type of event resulted in a lasting PVR increase as reflected in the new, lower flow. When the hypoxic stimulus was introduced an unstable situation was created. The vasoconstriction resulted in a zero flow condition in spite of unaltered pulmonary arterial pressure. When the hypoxic stimulus was withdrawn only a modest increase in flow occurred. In order to regain flow it was necessary to increase inflow pressure. Thus, it appeared

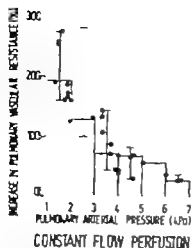


Fig. 3 Percentual increases in pulmonary vascular resistance caused by standardized periods of ventilation hypoxia in a group of rat lungs perfused with constant flow. Each point represents one response. The columns and bars indicate the median increase in vascular resistance and range of responses respectively for each 1 kPa rise in baseline (control) pulmonary arterial pressure.

as if a part of the vascular bed remained permanently closed.

The two above experiments are typical for the differences encountered when a standardized vasoconstrictor stimulus was introduced during the two modes of perfusion. Constant pressure perfusion gave a higher percentage rise in PVR than constant flow perfusion. This apparent advantage for quantitation of a vasoconstrictor response is however more than offset by the finding that (a) vascular closure often occurred making detection of graded responses impossible and (b) control situation was rarely regained.

When inflow pressure was set at gradually higher levels vascular closure could be avoided. The response however was then attenuated.

In Fig. 3 is presented 36 vasoconstrictor responses obtained in a group consisting of 17 lung preparations perfused at constant flow. Although the stimulus is standardized the vasoconstrictor effect varied from one preparation to another. This degree of inter-individual variation is well known both from studies on intact animals and man and from studies on isolated lungs (Hauge 1968c). The main point of the figure is however the effect of elevation in baseline pulmonary arterial pressure on the response to hypoxia. There is a clear although not very marked tendency for the response to decline as the pressure is increased. Fig. 4 shows the same relationship obtained in a group consisting

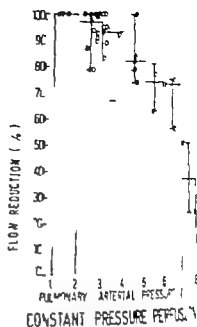
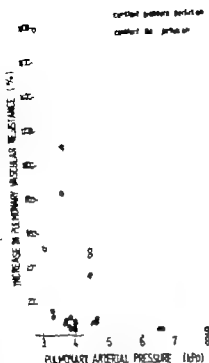


Fig. 4 Percentual pulmonary flow reduction in standardized periods of ventilation hypoxia in rat lungs perfused with constant inflow pressure at various levels of pulmonary arterial pressure. Each point represents one response. The columns and bars indicate the median flow reduction and range of responses respectively for each 1 kPa rise in baseline pulmonary arterial pressure.

of 13 lung preparations perfused at constant pulmonary arterial pressure. In all 48 responses evoked in this group. The choice of points directly to one of the main characteristics of constant pressure perfusion, namely a tendency to a zero flow situation to develop at inflow pressures upwards of 3 kPa whenever a vasoconstrictor stimulus is introduced, thus giving an extremely large increase in PVR. In comparison the increase in PVR in response to hypoxia at constant flow perfusion was only 190% obtained at the same range of baseline arterial pressures (1-7 kPa). At higher pulmonary arterial pressures the constant pressure technique of perfusion is also more pressure sensitive. This is demonstrated in Fig. 5 which is a composite diagram of the responses presented so far obtained in the two modes of perfusion. In order to avoid the values of Δ PVR during constant pressure perfusion the abscissa starts at a pulmonary arterial pressure of 3 kPa.

If we look at the pressure interspace at 1 kPa the median increase in PVR during constant pressure was 1103% (m. 93).



Percent increases in pulmonary vascular resistance by standardized periods of ventilation in two groups of rat lungs perfused with constant pressure technique and constant volume flow technique, respectively. The responses are plotted against either (control) pulmonary arterial pressure at (and) which the responses were obtained

flow 83% (range 43–144) which is significant. Also at higher pressure levels, e.g. 6–7 the difference in responses between the two % of perfusion is highly significant, the increase in PVR being 273% (range 122–296) and 28% (range 27–38) for constant pressure and constant perfusion respectively. The main conclusion drawn from Fig. 5 is however the relatively small influence of baseline-pressure elevation on responses obtained during constant flow as compared with the constant pressure perfusion technique.

In both modes of perfusion pulmonary arterial pressure fluctuations with amplitudes of about 0.4 kPa were observed in the control situation (no hypoxia). When the vasoconstrictor response was induced as reduction in flow these pressure variations became smaller. In two experiments an overflow system (see Methods) was closed and necessary flow-reductions were done by manual

adjustments of the pump-setting. The purpose was to maintain unaltered pressure-pulsations during the vasoconstrictor responses. Mean inflow pressure in these two preparations was 3.20 and 3.00 kPa, respectively. In the first preparation flow had to be reduced from 21 to 0.4 ml/min during hypoxia in the other from 18 to 0.5 ml/min and from 15 to 0.2 ml/min during two periods of hypoxia. Pulse pressure amplitudes were never less than 0.4 kPa. We found no systematic differences when we compared the vasoconstrictor responses obtained with and without closed overflow system. Neither with constant nor with falling pulse-pressure amplitude was control PVR fully regained after a period of hypoxia, as long as vasoconstriction was measured as reduction in flow.

What is the effect on the response to hypoxia of elevations in venous pressure? We have attempted to answer this question by two additional experimental procedures. Firstly by left atrial pressure elevations in two of the preparations perfused at constant inflow pressure. Secondly by comparing the responses obtained during series-perfusion of two lung preparations, one anterogradely and one retrogradely both at constant flow. A typical example of the first type of procedure is shown in Fig. 6. Pulmonary arterial pressure was maintained at 2.8 kPa, hence a fall in driving pressure and flow was induced when left atrial pressure was increased. The higher vascular transmural pressure which was established did not, however prevent a zero flow situation when the hypoxic stimulus was introduced. The same result was obtained when the pulmonary arterial and left atrial pressures were increased to 4.86 and 2.26 kPa, respectively. The other preparation tested in this manner was perfused at pulmonary arterial pressure of 3.19 kPa. In this preparation, flow reductions were 94% both

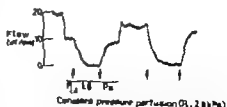


Fig. 6. Flow reductions caused by two periods of ventilation hypoxia (between arrows) in a pair of rat lungs perfused at constant pulmonary arterial pressure. The horizontal line indicates a period of left atrial pressure (P_{LA}) observation.

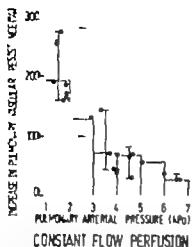


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of 13 lung preparations perfused at constant pulmonary arterial pressure. In all 48 responses evoked in this group. The choice of only different from that in the previous figure points directly to one of the main characteristics of constant pressure perfusion, namely a tendency to develop a zero flow situation at baseline pressures upwards to 3 kPa whenever a vasoconstrictor stimulus is introduced, thus giving an increase in PVR. In comparison the increase in PVR in response to hypoxia at constant flow perfusion was only 190% (obtained at same range of baseline arterial pressures (1 kPa)). At higher pulmonary arterial pressures the constant pressure technique of perfusion is also more pressure sensitive. This is demonstrated in Fig. 5 which is a composite diagram of the responses presented so far, obtained in both modes of perfusion. In order to avoid the values of Δ PVR during constant pressure perfusion the abscissa starts at a pulmonary arterial pressure of 3 kPa.

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higher vascular smooth muscle tension for a constrictor stimulus. This last factor may at least in part, explain why vascular closure occurs so rarely in isolated rat lungs if not countered by an increase in vascular transmural pressure.

It has been debated whether critical closure of vessels, as defined by Burton (1965), occurs at all in lungs. It has been pointed out that small intrapulmonary arteries, as defined by Harris & Burton (1977), contain relatively little smooth muscle and both an internal and external elastic laminae are thick enough to remove the instability but not so great in amount, can still leave a great lability for contractive stimuli, and allow graded responses. In some individuals and species however, severe hypoxia might induce a very high vascular smooth muscle tension so that active tension is able to win over the pressure. Regardless of the distending effect of elastic tissue the radius will tend to decrease and according to the Laplace relationship less tension will then be necessary to complete closure occurs.

Animals who respond with particular vigorous pulmonary vasoconstriction when exposed to hypoxia are prone to develop high altitude pulmonary edema. There is evidence which strongly suggests that the mechanism is similar to that of pulmonary edema which accompanies severe pulmonary embolism (Hultgren & Grover 1966). In pulmonary embolism occlusion of a major portion of the pulmonary vascular bed results in pulmonary hypertension. Areas of the pulmonary vascular bed which are not occluded may be subjected to abnormally high pressure and flow and unevenly distributed areas of pulmonary edema may be found. The distribution pattern of early high altitude pulmonary edema (Penaloza & Sline 1969). Pulmonary pressure as measured with wedged catheter on the arterial side is normal in these cases. The above considerations do not prove that vascular closure does occur at high altitude. An alternative explanation may be that the response to hypoxia is markedly although without actual vascular closure, from one lung region to another leading to regional variations in capillary hydrostatic pressure. Regional infinite high relative viscosity of blood in areas with low flow velocity due to vasoconstriction, is another possibility which has to be taken into account (Fischer et al. 1979). A final clarification of this question can only be made if the

dimensions of the responding vessels are measured directly.

The effects of active tension on the stress-strain properties of arteriolar smooth muscle have been studied directly *in vivo* in the frog mesentery (Gore 1972). The arteriole was stretched by various levels of distending pressure and vessel diameter was measured with the smooth muscle relaxed. The vessel was then stimulated to contract maximally (with noradrenaline) at various levels of initial stretch and the reduction in diameter noted. The response of the vessel was markedly dependent on initial length. Thus as the distending pressure increased from low to intermediate levels the ability of the arteriole to constrict actively increased markedly but then at higher initial stress (or higher transmural pressures) the ability to constrict gradually decreased again. This relationship is similar to that found in many other types of muscle.

In the present work we found only the right hand part of the above relationship: any rise in initial transmural pressure caused reduction in the response. Thus, the maximal response appeared to occur at a lower than normal pulmonary arterial pressure in the isolated rat lung. Since the response did not seem to be much reduced at the lowest levels of stress this will enhance the likelihood of critical closure due to active tension. The situation may be different in other species and possibly also in rat lungs *in situ*. The *in vivo* autoperfused lung lobe preparations in cat (Hauge & Staub 1969; Barer et al. 1970) did however often show a zero flow condition when ventilated with N_2 . An interesting observation mentioned briefly in the work of Benumof & Wahrenbrock (1975) is that in one dog a slight increase in the response to hypoxia was found as the left atrial pressure was elevated from 3 to 13 mmHg (0.40 to 1.73 kPa).

Individuals both human and animal with particular brisk responses to hypoxia may show less attenuation of the active constriction as the vascular diameter is reduced comparable with the response pattern found in isolated rat lungs, and experience vascular closure in some part of the lungs. The mechanism of high altitude edema as outlined above, seems to be present only in a few individuals when exposed to hypoxia. It is a rare complication, and it correlates well with the responsiveness of the pulmonary vascular bed to hypoxia.

Finally it could be argued that the observations reported in this article are due to differences in flow

when left atrial pressure was 0 and 0.93 kPa. Flow reduction was 89% when left atrial pressure was increased to 1.60 kPa.

In 14 series-perfused preparations (7 retrogradely and 7 anterogradely) baseline PVR and vasoconstrictor responses were measured in terms of inflow pressure. Mean baseline inflow pressure in the retro and antero groups were 1.46 kPa (range 1.20–2.00 kPa) and 1.33 kPa (range 0.93–2.13 kPa) respectively. The median increases in PVR due to hypoxia were 100% (range 54–146) and 112% (range 33–257%) respectively. Thus in spite of the large differences in left atrial and venous pressure in the retrogradely and anterogradely perfused lungs no significant differences were found as regards baseline PVR ($P=0.77$) and the vasoconstrictor response ($P=0.94$). In one single preparation perfused retrogradely at constant flow a pressor response as high as 3.86 kPa was obtained.

DISCUSSION

There are two types of information to be gained from this study. One is of a practical methodological kind and can be utilized when investigations of vasoactive substances and/or inhibitors are planned. The other type of information is of interest in its own right since it increases our knowledge about the behaviour of the pulmonary vasculature.

Reasonable demands to a test system are that the response to a given stimulus is reproducible and that a dose/response relationship can be established. Isolated vascular smooth muscle strips usually fulfill these demands but data obtained with this method are virtually impossible to interpret in terms of vascular resistance and flow deviations. Furthermore only relatively large vessels can be studied with this method. Isolated perfused organs in *casu* lungs have several advantages. Most workers have used a constant flow perfusion technique with inflow pressure as the dependent variable. As electromagnetic flowmeters have become generally available constant inflow pressure perfusion technique with flow as the dependent variable has gained in importance.

We have compared the practical usefulness of the two methods by using the vasoconstrictor response elicited by acute alveolar hypoxia as a model. We found as expected and in agreement with Benumof & Wahrenbrock (1975) that with rising baseline (control) vascular transmural pressure the vaso-

constrictor response was reduced. This was the case for both methods. Since during constant perfusion the response itself is measured in terms of pulmonary arterial pressure one might have expected that this would severely limit the use of this method for quantitative studies. When compared with the alternative technique it turned out however that the constant flow method was superior. Not only was it less sensitive to variations in inflow pressure but it also had another advantage. When the vasoconstrictor stimulus was removed the control situation was rapidly and completely regained making possible the application of a large number of constrictor stimuli under the same hemodynamic condition. It has been shown that this perfusion technique gives reproducible dose/response relationships (Bjertnes 1977) and that baseline pulmonary arterial pressure was within normal range.

In contrast whenever the hypoxic stimulus was applied under condition of constant pressure a permanent increase in vascular resistance was seen. In order to regain flow pulmonary arterial pressure had to be increased. At normal or slightly higher pulmonary arterial pressure the vasoconstriction induced with this stimulus rapidly led to vascular closure thus making graded responses impossible to study. Although arterial pressures total vascular closure was averted although a permanent rise in total vascular resistance usually occurred suggesting constriction of some part of the vascular bed. Exposure to hypoxia in isolated perfused lungs caused a change in vascular resistance constant pressure perfusion at all levels of baseline pressure gave responses to standardized periods of acute hypoxia. In order to demonstrate the presence or absence of hypoxic vasoconstriction this may therefore be the method of choice. For quantitative studies however the constant flow perfusion technique has several advantages. It is a more robust method allowing repeated application of the vasoconstrictor stimulus.

Benumof & Wahrenbrock (1975) found that the response was usually abolished when left atrial pressure was 25 mmHg (3.33 kPa) or pulmonary arterial pressure about 30 mmHg (4.01 kPa). In the present work the response was present at pulmonary arterial pressures upwards to 8 kPa. These factors can contribute to explain this difference: (1) the vascular dimensions are different in dogs and (2) the rat is a better responder than

Simultaneous determinations of metabolic and hormonal responses to heart rate, temperature and oxygen uptake during running in rats

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We have developed a new metabolism chamber including treadmill, for the study of exercising rats. The effects of work time, speed and inclination of running on \dot{V}_{O_2} , heart rate (HR), respiratory exchange ratio (R) and rectal temperature (T_{re}) were determined. During prolonged running \dot{V}_{O_2} , HR, R and catecholamine concentrations in arterial plasma drawn through chronically implanted catheters displayed an initial overshooting which did not diminish during the four times the rats within 10 days participated in experiments. When graded exercise was performed after a pre-test run of low intensity \dot{V}_{O_2} , HR, R and T_{re} increased with increasing running speed but did not vary with inclination. During 45 min of running, the plasma concentration of glucagon increased and that of insulin remained unchanged in spite of a marked increase in blood glucose. Conclusion: In exercise studies in rats it is necessary to introduce a 20 min pre-test period, during which unspecific stress responses disappear. This pre-test period cannot be replaced by 'habituation runs' on 3 preceding days. Furthermore, using rats, running speed rather than inclination has to be changed in order to establish physiologically significant differences in work intensity. The chamber makes it possible to carry out sophisticated studies of adaptations to exercise of a wide range of intensities in exercised rats and to relate the responses to \dot{V}_{O_2} .

Key words. Exercise test, adrenaline, noradrenaline, insulin, glucagon, blood glucose, homeostasis.

Rats are extensively used as an experimental animal in studies of acute and chronic adaptations to exercise. Nevertheless, there is a shortage of valid information about the relationship between work intensity and basic physiological parameters obtained at steady state in exercising rats. This is because it is difficult to evaluate work intensity by measurements of actual and maximal oxygen uptake and partly because the small size of the animal impedes sampling of blood tissues and neural signals. Studies of metabolic and hormonal responses to exercise in rats have generally been limited upon sampling of blood and tissues after the animal has stopped from handled anesthetized animals.

The aim of the present study was to develop an experimental model (apparatus and procedure) for steady state investigations of untrained rats during controlled exercise. The effects of work time, speed and inclination of treadmill running on oxygen up-

Abbreviations used. \dot{V}_{O_2} =oxygen consumption, \dot{V}_{CO_2} =carbon dioxide excretion, HR=heart rate, T_{re} =rectal temperature, R=respiratory exchange ratio, \dot{V} =flow rate of outlet air, $F_{I_{O_2}}$, $F_{I_{CO_2}}$, $F_{I_{N_2}}$ =decimal fractions of O_2 , CO_2 and N_2 , respectively in dry inlet air, F_{O_2} , F_{CO_2} , F_{O_2} =decimal fractions of O_2 , CO_2 and N_2 , respectively in dry outlet air, STPD=standard temperature and pressure, dry ATPD=satient temperature and pressure, dry NA=plasma concentration of noradrenaline, A=plasma concentration of adrenaline, b.w.=body weight.

pulsatility rather than the method of perfusion. In the constant pressure series inflow pressure pulsations tended to become smaller during vasoconstriction whereas in the constant flow series they tended to increase. The two experiments with closed overflow system and thereby unaltered pulse pressure suggest however that pressure pulsations are not a decisive factor. Results from in vivo autoperfused lung lobe preparations in cat (Barer et al 1970) support this conclusion. In spite of cardiogenic pulsations a zero flow condition often occurred during ventilation hypoxia.

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Tables including all the relevant hemodynamic data are available from the authors on request.

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$$\sqrt{STPD} \left(\frac{F_{O_2}}{F} F_{I_{O_2}} - F_{O_{2e}} \right)$$

$$\sqrt{dSTPD} \left(F_{CO_2} - \frac{F_{O_2}}{F} F_{I_{CO_2}} \right)$$

F_{CO_2} was determined as $I - F_{O_2} - F_{CO_2}$ calculated as

b

rate was determined by counting the number of breaths in 6

preparation

Wean rats had one half to two turns (about 2 ml) tail cut off by electrocoagulation in ether anaesthesia to avoid trauma of the tail during treadmill. No infections or growth retardation were observed. Furthermore, the amputation at the base of the gently scraped off in order to establish better contact between skin and tail electrodes.

The rats had catheter (PP 50 Polystan) inserted in common carotid artery in isoflurane-ether (Phar (0.01 ml per 100 g b.w.) anaesthesia. The following was found to be optimal resulting in 100% of its functioning more than 10 days: before operation the rat was tapered by pulling it over flame so that was very thin and flexible and an enlargement was 1.0 cm from the tip using ring of electrically heated chrome wire 3.0 cm of the heparine-saline filled U heparine per ml saline (0.9%) catheter was inserted in the artery and secured with ligature at each side enlargement. The tip was then freely floating in the artery. The catheter was led subcutaneously around the neck, the skin between the ears. After the operation vet (Pharmacia) (0.01 ml per 100 g b.w.) given as isoflurane-ether. The catheter was 2 with heparine-saline once 5 days later 10 days later. Then the rats were used for expts. They gained 17 (4-32) g (mean and range) in weight. They were housed in individual cages.

Rats were accustomed to run on 3 consecutive days each day at speeds up to 19 m/min. In the final run an air jet was activated to guide them. Not all rats were able to run but no rats were discarded.

general procedure

3 day of the actual expts. the rats were lightly ether anesthetized and for ECG-recordings clip with small pin was attached to each side of thorax after shaving the fur and applying electrode cream. One of the electrodes served as ground lead. The rats with already implanted catheter had saline filled catheter connected to the catheter by means of 2 cm of 23 gauge.

30 min before the ether anaesthesia those rats, which operated in exercise expts. were gently handled while tail electrodes were attached, the lubricated rectal inserted 4 cm beyond the anal sphincter and

taped to the tail, and the ECG-electrodes connected. The rats were then placed on the treadmill with the belt moving at speed of 10 m/min and an inclination of 0% in this way avoiding that the rats turned around and got themselves entangled in the wires.

8 rats (weight 209 (197-238) g in the first expt. weight gain 15 (-7+51) g during the experimental period (10 (6-15) days)) ran four times at an inclination of 0, 5, 10 and 15% respectively. The individual rat always ran at the same time of the day. The order of inclinations was systematically selected to minimize influence of habituation and training. Each exercise test was initiated by 31 run at a speed of 10 m/min and an inclination of 0%. Air flow F_{O_2} , F_{CO_2} , HR, T, box temperature and the temperature of the outlet air were determined in the 7th, 13th, 19th, 25th and 31st min of exercise. Then the inclination was set, and the speed was increased stepwise (within 0.5 min) with 5 m/min every 11 min. Recordings were made in the 11th min at each work intensity. At that time F_{O_2} and F_{CO_2} were constant. All rats were able to perform at a speed of 20 m/min at all inclinations and 7, 5, 7 and 4 rats of 25 m/min at an inclination of 0, 5, 10 and 15% respectively. Only one rat was able to run at a speed of 40 m/min.

The day after the last run each of the 8 rats was subjected to a swim in an air-tight plexiglas cylinder (19 cm d., 38 cm high) filled with water (33 °C and equilibrated with atmospheric air) to depth of 36 cm. V_{O_2} was determined using the same principle as described for the treadmill with an air flow of approximately 1000 ml (ATPD) min⁻¹. The rats swam in 6 min periods with increasing tail weights (0-11 g) until V_{O_2} levelled off or decreased.

In the morning after 1-2 h without access to food the 18 catheterized rats (weight 243 (220-262) g) either rested for 45 min or ran at an inclination of 0% 15 min at speed of 10 m/min followed by 30 min at speed of 14 m/min. Recordings were made in the 5th, 15th, 30th and 45th min of rest and exercise respectively (in rest expts. T was only measured immediately before the start of the experimental period). In the 5th, 15th and 30th min 0.7 ml of blood for catecholamine analysis was drawn from the catheter into heparinized tubes containing 1.4 mg EDTA and 1.4 mg ascorbic acid discarding the saline filling the catheter. The blood removed was replaced by blood obtained in heparinized syringe by heart puncture of donor rat immediately before the expt. Thereafter the catheter was refilled with saline. In the 45th min 0.7 ml of blood was drawn for catecholamine analysis and then 2.5 ml was drawn for analysis of glucose and lactate, and for analysis of plasma insulin and glucagon (2 ml into acid test tubes containing 100 µl Trasylol (Bayer)). Adrenaline (A) and noradrenaline (NA) were determined by radioenzymatic method, and glucose (hexokinase) and lactate by enzymatic spectrophotometric methods. Insulin and glucagon were determined by radioimmunoassays using rat insulin and pork glucagon as standards.

Statistical analysis of the data was performed using the Wilcoxon rank sum test for paired and the Mann-Whitney rank sum test for unpaired data. Correlation coefficients were calculated and compared according to Siedecor & Cochran (1963).

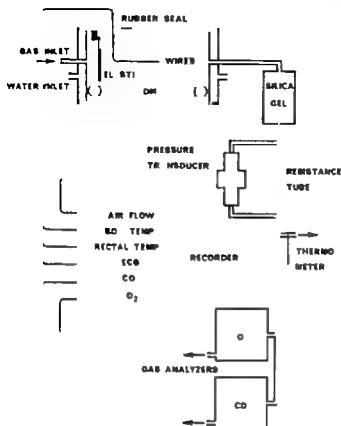


Fig. 1 Schematic diagram of metabolism chamber and auxiliary equipment. EL. STIM. displacable plate and microswitch

take (V_{O_2}), heart rate (HR), respiratory exchange ratio (R) and rectal temperature (T_R) were determined in order to establish an experimental procedure for attainment of valid informations about responses to different levels of exercise in rats. Since levelling off of oxygen uptake was not found we evaluated if the highest oxygen uptakes measured during running were really the highest obtainable during exercise by comparing them to oxygen uptakes measured during forced swimming. Finally chronically implanted arterial catheters were used for blood sampling from unanesthetized rats at rest and during running making it possible optimally to measure metabolic and hormonal responses to exercise.

MATERIALS AND METHODS

Description of apparatus (Fig. 1)

The system consists of a tiny motordriven treadmill the speed of which can be continuously varied between 0 and 80 m/min. A velocimeter with digital display makes it possible to adjust the speed accurately. The motor is

placed outside and the treadmill inside a double metabolism chamber (inside dimension 29 cm \times 13 cm) the inclination of which can be corrected from 0 to 15° allowing studies during uphill running possible to establish a flow of water between the (space 1900 cm³) thus regulating the temperature in expts. where extreme ambient temperature is required. A thermistor 1 cm below the lid measures temperature in the box.

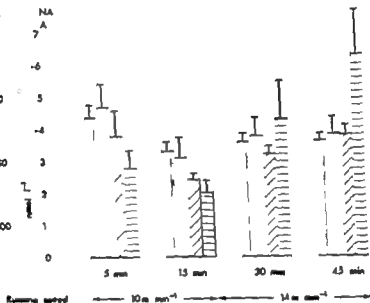
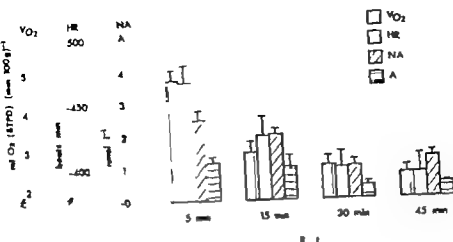
An adjustable flow of tank gas (all the proper atmospheric air) is led into the box at the rear of the treadmill. The outlet air passes through coarse 5' for dehydration and further through a resistance (13 gauge 29 cm) over which the pressure is measured by a strain gauge differential pressure transducer. The system is calibrated using known air flow and serves as a pneumotachograph. Flow rate is set to approximately 1500 ml (ATPD) min. in expts. and in the initial 6 min of rest expts. the flow rate of approximately 1000 ml (ATPD) is used from the 7th min onward in rest expts. that resulting in box pressures 0.8 and 0.5 kPa above metric pressure respectively and in a CO₂ concentration in the box air below 1% immediately after the inlet tube 600 ml (ATPD) min. is withdrawn from a 1-l gas analysis whereas the excess air passes a flow meter. An oxygen analyzer (Applied Electrode S-3A oxygen analyzer with a N 22M oxygen sensor a R 1 flow control) and a carbon dioxide analyzer (man LB-2) are connected in parallel. The air flow to the analyzers are 100 and 500 ml (ATPD) respectively yielding the optimal accuracy for both gases. The gas-analyzers are calibrated before each rest tank gases with approximately 21 and 17% O₂ and 4% CO₂. All tank gases are analyzed by the flow technique. The electronic analyzers are stable for 1 h.

The rats are encouraged to run by mild electrical stimulation. A plexiglas ring (10 cm i.d. 14 cm o.d.) with small cylinder head machine screws encloses the tail. If the rat stops running it bumps into a displacable plate at the rear end of the treadmill. A microswitch is turned off and the rat receives a constant current through the tail electrodes as long as the plate is displaced. The voltage can be varied from 0 to 40 V.

The wires for the tail electrodes enter the box at the lid at the rear end of the treadmill together with wires for the box thermistor for a rectal thermistor for two small banana plugs used for ECG-recording. A disposable pierceable rubber seal makes it possible for catheters air tight through the lid. The outputs for the pneumotachograph, the gas-analyzers and the rectal thermistor are recorded on a six-channel recorder (Channel 370) using locally constructed amplifiers. The recorder is calibrated to give deflections of 1 cm per ml (ATPD) min., 1 cm per 0.2% of O₂ and CO₂, 0.2°C (rectal thermistor) and 1 cm per 0.1°C thermistor. The ECG-signal is recorded on an Ultralette 3651 at a paper speed of 75 cm/min.

Calculations

V_{O_2} and V_{CO_2} were calc.



$\dot{V}O_2$, HR, noradrenaline (NA) and adrenaline (A) during 45 min of rest or running at an inclination of 8%. Values are \pm S.E. for 9 rats.

Rectal temperature

Rectal temperature in the box was at the start of expts. 23.0 (19.8–28.0) °C and it increased 1.8 (3.5) °C during running and 0.6 (0.2–1.4) °C in expts.

31 min of exercise (Fig. 2)

$\dot{V}O_2$ decreased ($P < 0.05$) from the 7th to the 31st min after it remained unchanged. HR and R

decreased from the 7th to the 19th min of exercise ($P < 0.05$) whereafter they remained unchanged. T did not change significantly during the 31st min of running.

Neither $\dot{V}O_2$, HR, NA nor T changed significantly from the first to the fourth time the rats participated in exercise expts. Comparison of the two regression lines for HR (y) versus $\dot{V}O_2$ (x) calculated at the 7th ($y = 365 + 19x - 31$) and 31st min ($y = 333 + 20x$

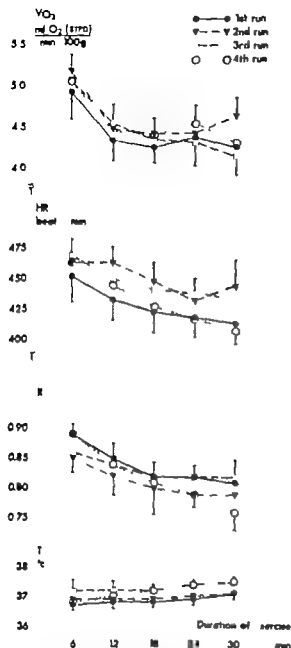


Fig. 2 $\dot{V}O_2$, HR, R and T during four runs on separate days at a speed of 10 m/min and an inclination of 0%. Values are mean \pm S.E. for 8 rats.

RESULTS

Kinetics

A series of expts. was performed to evaluate the mixing of the air in the metabolism chamber. 100% N_2 was introduced in the chamber at different flow rates and F_{O_2} was registered continuously. When the treadmill belt was not moving a biexponential wash out curve occurred ($k_1=0.77$ and $k_2=0.28$ min $^{-1}$ at a flow rate of 1475 ml (ATPD) min $^{-1}$). But when the belt was moving at a speed of 10 m/min $^{-1}$ the wash out curve was

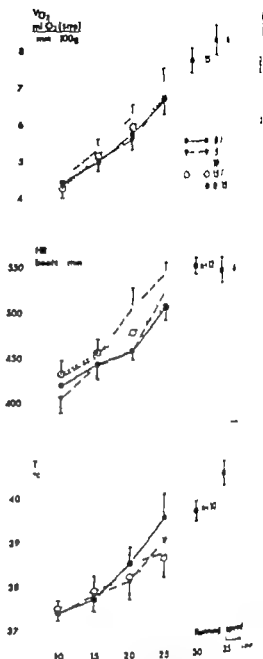
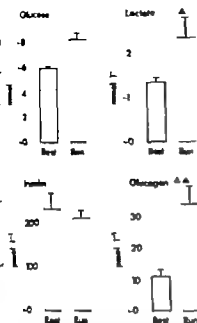


Fig. 3 $\dot{V}O_2$, HR and T as a function of running speed and inclinations. Values are mean \pm S.E. for 8 rats.

monoexponential with a T_1 which at the flow rate used in the expts. agreed exactly with the T_1 calculated as

$$\ln V \left[\frac{\text{volume of air in chamber (7900 cm}^3\text{)}}{\text{flow rate}} \right]$$

indicating complete mixing of the air in the chamber. When the rat volume is taken into account T_1 can be calculated to 1.2 min using above formula at a flow rate of 1475 (ATPD) min $^{-1}$.



Blood glucose and lactate concentrations and insulin and glucagon concentrations in the 45th min of running (15 min at running speed of 10 m/min followed by 30 min at 14 m/min) at an exchange rate of 6%. Values are mean \pm S.E. for 9 rats. Δ denotes $P < 0.05$.

lactate concentrations (Fig. 4). Accordingly the peculiar finding of relatively high V_{O_2} at low speeds in the beginning of the graded exercise test probably was an effect of catecholamines released as an excitement reaction to handling and a new environment. Increases in the plasma concentrations of catecholamines upon handling and rest have previously been found in the rat (Poppe et al. 1977; Kvetnansky et al. 1978). The initial shooting in V_{O_2} and HR did not diminish in the four times the rats within a period of 10 min participated in exercise tests (Fig. 2). A similar overshooting was found in rats studied in (Fig. 4). V_{O_2} initially being equal to $V_{O_{2s}}$ during steady state running at about 13 m/min. As a consequence of the above findings we found it necessary to begin each exercise test with a 31 min habituation period. A habituation period of slow running was chosen rather than a period of rest, as the rats at rest would get themselves entangled in the wires. Furthermore the switch on of the treadmill probably would elicit excitement

reactions. After the introduction of a pre-test period the responses to exercise were as expected. V_{O_2} , HR, R and T increased with increasing speed of running (Fig. 3). In the light of our findings it seems likely that many previous measurements carried out in exercised rats reflect excitement reactions as well as responses to exercise, as this was probably the case in a recent study in which cardiovascular parameters were measured after 5 min of running in rats which had two arterial catheters implanted 3 h in advance and which had no prior treadmill experience (Flam et al. 1979).

At the end of each graded exercise test the rats ran at a speed which they were only able to cope with for a period of time which as a consequence of the response time of the whole system, was too short to allow measurement of the actual V_{O_2} . The response time might be shortened by an increase in flow rate but then the accuracy of the readings of the gas analyses would decrease. However during the few minutes at the highest speed F_{O_2} and F_{CO_2} changed to values lower and higher respectively than those obtained at lower speeds. This indicates that the highest measured V_{O_2} was not maximal, a supposition supported by the fact that V_{O_2} and HR did not level off at the highest speeds (Fig. 3). However the highest V_{O_2} which we were able to measure during running was probably nearly maximal. This is so since it was higher than the highest V_{O_2} measured during forced swimming, the latter agreeing with previously reported values (McArdle 1967). Furthermore it was identical to maximal V_{O_2} defined by a levelling off criterion, in untrained, running (Wilson et al. 1978) or cold exposed (Rosenmann & Morrison 1974) rats.

T_{re} was higher during running than at rest and increased with running speed in these experiments (Fig. 3) in which the ambient temperature was within the zone of thermoneutrality as defined for rats (Poole & Stephenson 1977). It could be argued that the lack of a part of the tail might render heat loss difficult and thus augment the T_{re} response to exercise. However it can be calculated that the removed part of the tail at the most amounted to about 6% of the total non-evaporative heat dissipation capacity in the rat (Rand et al. 1965). Furthermore when core temperature during exercise exceeds 39°C evaporation of saliva spread over chest and forelegs probably aids in heat dissipation, and the tail accordingly accounts for a smaller fraction of the total heat loss than during non-evaporative

Table 1 *Correlation coefficients between \dot{V}_{O_2} , HR and running speed at inclinations of 0, 5, 10 and 15%*

| Inclination | 0% | 5% | 10% | 15% |
|---------------------------|--------------|--------------|--------------|--------------|
| \dot{V}_{O_2} and speed | 0.80 (39) | 0.88 (34) | 0.80 (35) | 0.74 (32) |
| HR and speed | 0.68 (32) | 0.86 (23) | 0.76 (25) | 0.83 (25) |
| HR and \dot{V}_{O_2} | 0.76 (31) | 0.85 (23) | 0.78 (25) | 0.79 (25) |
| T_R and speed | 0.74 (31) | 0.73 (25) | 0.71 (22) | 0.69 (29) |
| T_R and \dot{V}_{O_2} | 0.82 (31) | 0.82 (25) | 0.86 (22) | 0.66 (29) |

All correlation coefficients are statistically significant ($P < 0.001$). Correlation coefficients obtained at different inclinations are not significantly different from each other. Number of observations is shown in parentheses.

$n=31$) revealed that the regression lines may be regarded as parallel but with different intercepts ($P < 0.001$) indicating that in the 7th compared to the 31st min HR is relatively more increased than \dot{V}_{O_2} .

Effects of running speed

\dot{V}_{O_2} and HR increased with increasing running speed ($P < 0.05$) at all inclinations (Fig. 3). Positive correlations between \dot{V}_{O_2} and speed, HR and speed and between HR and \dot{V}_{O_2} ($y = 98 + 30x$, $n = 135$) were found at each inclination (Table 1). The highest measured \dot{V}_{O_2} and HR were 8.6 ± 0.3 ml O_2 (STPD) (min 100 g)⁻¹ (mean \pm S.E., $n = 8$) and 556 ± 16 beats min⁻¹ ($n = 8$) respectively.

At the end of the initial 31 min period the R value was 0.81 ± 0.02 ($n = 23$). At a running speed of 25 m min⁻¹ R increased to 0.84 ± 0.01 ($n = 23$) ($P < 0.07$). A positive correlation between R and running speed was found ($r = 0.36$, $P < 0.001$). The highest measured R was 0.97 ± 0.02 ($n = 8$).

T_R increased with increasing running speed at all inclinations (Fig. 3). Compared to the values obtained at a speed of 10 m min⁻¹ the increase was significant at 20 m min⁻¹ ($P < 0.05$). Positive correlations between T_R and speed and between T_R and \dot{V}_{O_2} were found at each inclination (Table 1). The highest measured T_R was $40.0 \pm 0.4^\circ C$ ($n = 8$).

Effects of inclination

No significant differences in \dot{V}_{O_2} , HR, R and T_R were found when comparing measurements at different inclinations and identical speeds obtained in

expts carried out on different days (Fig. 4). Furthermore when comparing values obtained at 31st min of pre-test running at a speed of 10 m min⁻¹ and an inclination of 0% to values obtained 10 min later at the same running speed and inclination of 5, 10 or 15% no influence of the inclination on these parameters could be demonstrated.

\dot{V}_{O_2} during swimming

The highest measured \dot{V}_{O_2} during running was always higher (76 (3–59) %) than the highest measured \dot{V}_{O_2} during swimming (6.9 ± 0.2 ml (STPD) (min 100 g)⁻¹).

Rest (Fig. 4)

\dot{V}_{O_2} , HR and the plasma concentrations of NA decreased during the initial 30 min of rest ($P < 0.01$) and then remained unchanged. \dot{V}_{O_2} correlated with NA ($r = 0.66$, $P < 0.001$) in the 30 min of rest. T_R was 36.7 (35.9–37.9) $^\circ C$.

Metabolic and hormonal responses to exercise

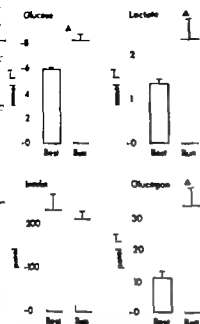
At comparable times A and NA were higher during prolonged running than at rest ($P < 0.05$) (Fig. 1) and NA ($P < 0.05$) decreased during the 3rd min of prolonged running and then increased ($P < 0.02$) with \dot{V}_{O_2} (Fig. 4). During the first 10 min of running A ($r = 0.55$, $P < 0.05$) and NA ($r = 0.40$, $P < 0.10$) correlated with \dot{V}_{O_2} .

Compared to the values obtained in the 4th min of rest blood glucose and lactate concentrations, the plasma glucagon concentration increased during running whereas plasma insulin concentration remained unchanged (Fig. 5).

DISCUSSION

In the present paper a metabolism chamber for study of running rats is described. A procedure for attainment of valid information about response to exercise in the rat was developed and used to characterize some metabolic and hormonal actions.

In our preliminary expts we found no surprise that \dot{V} decreased with increasing speed during an uninterrupted graded exercise test. Further investigations showed that \dot{V}_{O_2} , as HR, HR/ \dot{V}_{O_2} and R decreased during the first min of exercise at a constant work load. These changes were accompanied by decreasing F



Blood glucose and lactate concentrations and insulin and glucagon concentrations in the 45th min of rest or running (15 rats at running speed of 10 m/min followed by 30 rats at 14 m/min at an incline of 8%). Values are mean \pm S.E. for 9 rats. Δ denotes $P < 0.01$.

volatase concentrations (Fig. 4). Accordingly the peculiar finding of a relatively high V_{O_2} at low speeds in the beginning of the graded exercise test probably was an effect of catecholamines released as an excitement reaction to handling and a new environment. Increases in the plasma concentrations of catecholamines upon handling and rest have previously been found in the rat (Popal 1977; Kvetnansky et al. 1978). The initial shooting in V_{O_2} and HR did not diminish during the four times the rats within a period of 10 min participated in exercise experiments (Fig. 2). A similar overshooting was found in rats studied at rest (Fig. 4). V_{O_2} initially being equal to $V_{O_{2R}}$ during steady state running at about 13 m/min. As a consequence of the above findings we found it necessary to begin each exercise test with a 31 min habituation period. A habituation period of slow running was chosen rather than a period of rest, since the rats at rest would get themselves engaged in the wires. Furthermore, the switch on of the treadmill probably would elicit excitement

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T was higher during running than at rest and increased with running speed in these experiments (Fig. 3), in which the ambient temperature was within the zone of thermoneutrality as defined for rats (Poole & Stephenson 1977). It could be argued that the lack of a part of the tail might render heat loss difficult and thus augment the T response to exercise. However it can be calculated that the removed part of the tail at the most amounted to about 6% of the total non-evaporative heat dissipation capacity in the rat (Rand et al. 1965). Furthermore when core temperature during exercise exceeds 39°C, evaporation of saliva spread over chest and forelegs probably aids in heat dissipation, and the tail accordingly accounts for a smaller fraction of the total heat loss than during non-evaporative

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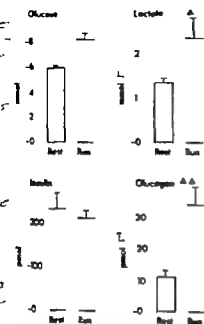
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Compared to the values obtained in the 30 min of rest blood glucose and lactate concentrations increased during running, whereas plasma insulin concentration remained unchanged (Fig. 5).

DISCUSSION

In the present paper a metabolism chamber study of running rats is described. A procedure for attainment of valid information about respiration during exercise in the rat was developed and used to characterize some metabolic and hormonal changes.

In our preliminary expts we found it surprising that \dot{V}_{O_2} decreased with increasing speed during running during an uninterrupted graded exercise test. Further investigations showed that \dot{V}_{O_2} , as HR, HR/ \dot{V}_{O_2} and R decreased during the first 10 min of exercise at a constant work load. These changes were accompanied by decreasing plasma



Blood glucose and lactate concentrations and insulin and glucagon concentrations in the 45th rest or running (15 min at running speed of 10 m/min followed by 30 min at 14 m/min at an inclination of 8%). Values are means \pm S.E. for 9 rats. Δ denotes $P < 0.01$.

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T_{re} was higher during running than at rest and increased with running speed in these expts. (Fig. 3), in which the ambient temperature was within the zone of thermoneutrality as defined for rats (Poole & Stephenson 1977). It could be argued that the lack of a part of the tail might render heat loss difficult and thus augment the T_{re} response to exercise. However it can be calculated that the removed part of the tail at the most amounted to about 6% of the total non-evaporative heat dissipation capacity in the rat (Rand et al. 1965). Furthermore when core temperature during exercise exceeds 39°C evaporation of saliva spread over chest and forelegs probably aids in heat dissipation, and the tail accordingly accounts for a smaller fraction of the total heat loss than during non-evaporative

conditions (Gollnick & Ianuzzo 1968; Poole & Stephenson 1977). The supposition that heat dissipation was not severely impeded is supported by the fact that T_{re} at rest and during exercise was slightly lower than previously reported for rats at similar ambient temperature (Gollnick & Ianuzzo 1968). However, this discrepancy may also to some extent be due to the fact that T_{re} had not always plateaued when read in the present expts. At rest also differences as regards the distance from the anal sphincter at which T_{re} is measured (4 cm in the present vs. 5.5 cm in the cited study) may explain the diverging T_{re} values (Lomax 1966). The relationship between site of measurement and T_{re} in the rat during exercise is unknown.

At constant speed of running treadmill inclination did not influence \dot{V}_{O_2} , HR, R and T_{re} in the present expts. This supports the conclusion that running uphill involves a relatively smaller increase in energy expenditure over horizontal running for small than for large animals (Taylor et al. 1972).

During prolonged exercise the plasma concentration of glucagon increased and that of insulin remained unchanged in spite of a marked increase in blood glucose concentration (Fig. 5). We have previously found a similar pattern in swimming rats (Richter et al. 1979). However, at that time the concentrations were measured after termination of exercise and it could not be excluded that a post exercise change had occurred. From studies carried out in dogs it has been claimed that during prolonged exercise the plasma glucose concentration is a precisely regulated variable which is kept constant in spite of large increases in glucose production and utilization (Vranic et al. 1976). It appears from the present study that in the rat the blood glucose concentration may indeed increase during prolonged exercise, indicating that at least in this species hepatic glucose production is not accurately adjusted to peripheral glucose utilization on a moment to moment basis during exercise.

In conclusion, the present study has shown that in order to carry out work physiological investigations in rats it is necessary to introduce a 70 min pre-test period during which unspecific stress responses to handling and a new environment disappear. This pre-test period cannot be replaced by 'habituation' runs on three preceding days. Furthermore, using rats running speed rather than inclination has to be changed in order to establish physiologically significant differences in work in-

tensity. Taking these findings into account the developed metabolism chamber makes it possible to carry out sophisticated studies of metabolic, hormonal and cardiovascular adaptations to exercise intensities almost up to maximal aerobic capacity in untrained rats and to relate the responses to uptake.

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Blood flow in arteries determined transcutaneously by an ultrasonic doppler velocitymeter as compared to electromagnetic measurements on the exposed vessels

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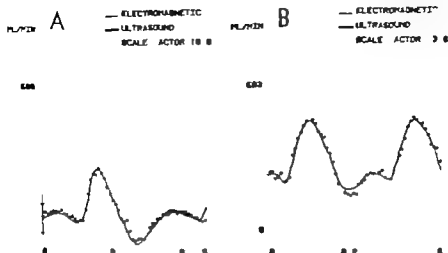
We have compared the instantaneous mean blood velocity measured transcutaneously from total cross-sections of arteries in dogs by the use of a new pulsed heterodyne ultrasound doppler equipment, with instantaneous blood flow as measured simultaneously by an electromagnetic flowmeter applied on the exposed vessels. The experiments were performed on the common carotid and the femoral arteries in a variety of flow situations, always with a close fit between the two curves. The results show that the output from the ultrasound equipment is proportional to flow

early 1960's Rushmer and collaborators described an ultrasonic doppler instrument which measures blood velocities through the skin (Rushmer et al. 1966). Theoretically the doppler signal from such an instrument should contain information about all velocities present in the vessel. All parts of the cross-sectional area of the vessel are illuminated by a soundfield of constant intensity. The back-scattered signal should also contain information about the relative amount of blood moving at each of these velocities. Thus in their papers, Rushmer and collaborators indicated the possibility of non-invasive volume flow measurements by means of a doppler instrument. Many attempts have been made to improve the existing instruments (see Woodcock 1975 for references). However the volume flow measurement has not yet been reached. This article presents an attempt in the direction of volume flow measurements.

We have tried to improve the existing ultrasound

equipment in three different ways. (1) The ultrasound probes have been carefully designed built and then measured to ensure an approximately constant sound-field with sufficient width. (2) A new doppler instrument has been constructed (Hartelund & Eriksen 1980) with sharp filters in order to remove stationary echoes and doppler frequencies caused by movements of the vessel wall without seriously affecting doppler frequencies caused by the moving blood. This instrument has a heterodyne audio output. (3) All estimates (e.g. of the mean doppler frequency across the vessel lumen) are made from frequency spectra which are calculated from the heterodyne audio output in a computer system (Wille 1977). Thus biased estimators such as the zero-crossing estimator are avoided, and it is easy to make the estimators robust against noise.

The aim of our project so far has therefore been to estimate the true mean doppler frequency across the vessel lumen and its variation with time. To



Comparison of the electromagnetic flowrecording and the best fitted ultrasound blood velocity measurements to different vessels in one dog. (A) Femoral artery 6 MHz. (B) Common carotid artery 6 MHz. In both panels vertical axis is time in s, and the horizontal axis is flow in ml/min as determined by the electromagnetic flowmeter below the horizontal axis correspond to backflow. The two panels were obtained from the computer by means of a dot plotter. The pulse frequency was little lower during the femoral recording than during the carotid recording. Assuming measurement angle of 45° scale factor 10.0 corresponds to internal diameter 2.48 mm, and scale factor 3.8 corresponds to internal diameter 2.97 mm.

Velocities towards the transducer cause frequency shifts above the chosen base-line and velocities away from the transducer cause frequency shifts below the chosen base-line frequency. UNIDOP contains 12 pole low-pass Butterworth filters adjustable to four different sampling frequencies with break point at 40 % of the sampling frequency. In addition one can choose from among three high-pass Butterworth filters with break frequencies at 200 Hz, 400 Hz and 700 Hz. These filters will eliminate frequencies caused by vessel wall movements and other disturbances.

The velocitymeter is connected to an analog real-time compression spectrometer in our set-up. It is possible to see the spectrum on a scope simultaneously while hearing the audio signal from the loudspeaker. One of the real time spectrum is to adjust the amplitude of the audio signal to get rid of most of the noise without losing too much of the information-containing signal. Another use of the spectrum is to discriminate between vessels depending on characteristic shapes of their spectra.

When the audio signal for later calculations, the UNIDOP is connected to a stereo cassette tape recorder (Akai CX-706 D). The doppler signal is recorded on one channel and the ECG and comments are recorded on the other channel. Both the tape recorder and the velocitymeter is connected on-line to a NOVA computer system. For practical reasons we usually store the recordings on magnetic tape and play the tape back for later calculations in the computer. The computer may calculate the spectrum as quickly as every 8 ms, using fast Fourier transform (FFT), and also the mean velocity from each spectrum (M77).

Now we want to measure the relative intensity of all vessels present in the whole cross-section of the vessel.

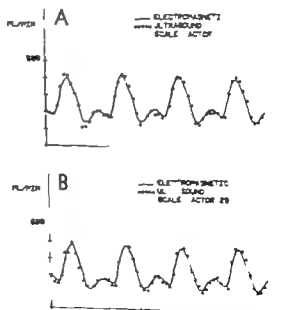


Fig. 3. Ultrasound blood velocity measurements as compared to electromagnetic flow recordings. (A) Common carotid artery 6 MHz. (B) Common carotid artery 1.5 MHz. Axes and symbols as in Fig. 2. A new mean doppler frequency is calculated each 20 ms. In panel A scale factor 14.3 corresponds to internal diameter 2.96 mm and in panel B scale factor 29.5 corresponds to internal diameter 3.36 mm.

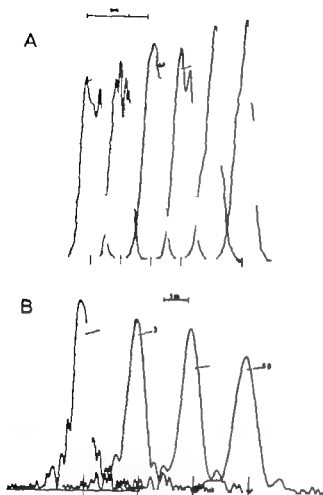


Fig 1 Sound fields emitted from the two ultrasound transducers. (A) Intensity distributions in front of the 6 MHz ultrasound transducer as measured normally to the beam axis in 6 different distances from the crystal surface in a water tank. The intensity was measured by a small ceramic crystal mounted on the tip of a needle. The effective diameter of the probe was 1.4 mm. Horizontal axis: distance across the field. Vertical axis: sound intensity. The scale is arbitrary, but is the same for all distributions. The 6 distributions are displaced along the horizontal axis for clarity. (B) Intensity distributions in front of the 1.5 MHz ultrasound transducer. Axes etc. as in A.

convert this mean doppler frequency to mean blood velocity either an a priori knowledge or a measurement of the angle between the sound beam and the velocity vector is necessary. The instantaneous blood flow through the vessel is equal to the mean blood velocity multiplied by the cross-sectional area of the vessel. Thus knowledge of or measurement of both the angle and this area are necessary to convert the mean doppler frequency to volume flow. The problems connected with measurements of the angle and of the cross-sectional area have not been approached in this study. However, in situa-

tions where the measurement angle and the cross-sectional area of the vessel can be varied approximately constant, changes in the Doppler frequency with time (for example during cardiac cycle) should be proportional to the flow. In the present study we have compared mean doppler frequencies obtained transcutaneously by use of our equipment in dogs with flow as measured simultaneously by an electromagnetic flowmeter applied on the exposed vessels.

METHODS

Surgical and experimental procedures

The experiments were performed on 3 seven-month-old golden retriever dogs (weight approximately 11 kg). The animals were anesthetized with i.v. injection of pentobarbital (Nembutal). During the experiment, ECG was recorded by needle-electrodes, and blood pressure by means of an intra-arterial catheter. The femoral vessels were exposed by short transversal sections in the skin. The electromagnetic flow probes were applied in a free-dissected segment of the vessel. The ultrasound was aimed at the vessel 1–2 cm upstream from the transducer. The ultrasound probe was held by hand at an angle to the vessel approximately 45°. Based on knowledge of the vascular anatomy of the dog, we tried to choose a segment of the femoral artery without any branches between the two measurement areas. The common carotid vessels were exposed in the same manner 5 cm beneath the jaw. However, since the common carotid does not have any branches, the ultrasound measurement could be performed both cranially, near the power of the electromagnetic flow probe, and closer to the heart, where the vessel runs deeper.

The ultrasound doppler velocimeter

The doppler velocimeter UNIDOP used in the investigation and part of the computer system for data analysis have been described in detail elsewhere (Hatteland & Eriksen 1980; Wille 1977). Velocity measurements can be obtained from most vessels in the body as long as the sound beam does not hit bone or gas (lungs or parts of the intestines). To take measurements on deep vessels down to the depth of 10 cm, a frequency 1.5 MHz is used, which gives a good penetration. Recording from superficial vessels < 5 cm one uses 6 MHz, which gives a better resolution. In the present investigation 6 MHz was used on the femoral artery and on the cranial part of the common carotid artery, while 1.5 MHz sound was used on the central part of the common carotid artery. The frequencies can be operated either in a pulsed or in a continuous wave mode. The pulsed mode was used in the present investigation. The pulse lengths were 0.5–1.0 ms.

UNIDOP uses heterodyne detection to discriminate between forward and backward flow. The base-line

2. Repeated doppler velocity measurements common carotid artery as compared to electromagnetic flow measurements

nasal probe was removed completely between consecutive observations no. 5 and 6 were obtained closer to the heart than the first four

| EM-flow (ml/min) | Scale factor | Doppler flow (ml/min) | Calculated radius (mm) |
|---------------------|-----------------|-----------------------------|------------------------------|
| 226.2 | 14.5 | 218.3 | 1.49 |
| 212.2 | 14.8 | 206.5 | 1.51 |
| 238.3 | 13.8 | 234.6 | 1.46 |
| 233.4 | 14.6 | 228.7 | 1.50 |
| 202.1 | 17.0 | 200.6 | 1.6 |
| 190.2 | 17.9 | 184.5 | 1.66 |

A the cross-sectional area of the vessel. If both cross-sectional area A and the measurement angle θ assumed in \sin be constant, the mean doppler frequency f_d the instantaneous flow should be proportional. As approximation we assume that these conditions are θ during one pulse cycle. Consequently instantaneous flow and instantaneous mean blood velocities were compared during corresponding cardiac cycles. The comparison procedure has been as follows. Parts of recordings where both the electromagnetic flowmeter transducer velocimeter the arterial pressure measurements, and the ECG were operating properly were used. From the ultrasound recording, the mean doppler velocities were calculated each 8 ms through one or cardiac cycles as described in Wille (1977). The corresponding recording from the electromagnetic velocity was sampled at exactly the same points in time. The computer then determined the proportionality factor k necessary to obtain the best fit between the velocity recording and the flow recording. A least mean absolute deviation between the two curves was the criterion for k . In theory the proportionality factor is function of cross-sectional area of the vessel and of the measurement angle. Assuming an angle of 45° and assuming the cross-section of the vessel it is possible to calculate internal diameter of the vessel corresponding to best fit between the two curves.

RESULTS

Figure 2 shows the results obtained in two vessels in the same dog. An emitting frequency of 6 MHz was used for the ultrasound measurements both on the femoral artery and on the common carotid artery. Figure 3 shows similar pairs of curves. Both panels represent measurements on the common carotid artery. The upper panel displays ultrasound

measurements obtained near the flow transducer by means of 6 MHz ultrasound. The lower panel displays measurements obtained deeper on the neck near the thorax by 1.5 MHz ultrasound. A and B are not simultaneous recordings, but were obtained from the same dog.

Fig. 4 shows the results obtained from a femoral artery before (A) and after (B) an intra arterial injection of papaverin (20 mg). In Fig. 4A the average flow during one cardiac cycle is 45.5 ml/min and in Fig. 4B the corresponding flow is 110.0 ml/min.

Figures such as those displayed in Figs 2, 3 and 4 were obtained in all three dogs in a variety of flow situations. Towards the end of each experiment, either papaverin or bradykinin was injected with large vasodilatation as the result. The fit between the electromagnetic flow and the ultrasound blood velocity was equally good in these flow situations.

A few longer series of recordings were obtained for statistical purposes.

In some series the ultrasound transducer was kept in position continuously for more than half a minute. Table 1 shows the results from one such series. The best fit to the electromagnetic flow-recording was obtained individually for each heart beat. The table illustrates the typical degree of scatter between successive cardiac cycles.

In other series the ultrasound transducer was removed completely from the skin while the investigator was busy with other tasks. Afterwards the transducer was aimed in at the vessel again. This procedure was repeated a number of times. Table 2 shows the results from one such series.

DISCUSSION

The results show that a very good fit can be obtained between our ultrasound blood velocity measurements and the electromagnetic flow measurements. However as long as the good fit is obtained partially by means of the arbitrary scale factor we are somewhat reluctant to draw too firm conclusions. Clearly the results would have been more convincing if both the cross-sectional area and the measurement angle had been measured during the experiment and the subsequent fit was obtained without any curve fitting. However precise measurements of the internal cross-sectional area of a vessel under different physiological and pharmacological conditions is research project in its

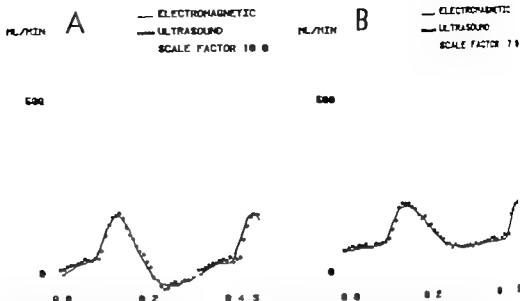


Fig. 4 Ultrasound blood velocity measurements as compared to electromagnetic flow recordings. (A) Femoral artery 6 MHz. (B) Femoral artery 6 MHz, after injection of papaverine. Scale factor 10.0 corresponds to internal diameter 2.20 mm. Scale factor 7.9 corresponds to internal diameter 2.20 mm. Blood pressure in A was 156/136 mmHg, in B 144/114 mmHg.

we use transducers which produce a sound field slightly broader than the diameter of the vessel and the sound field has approximately constant intensity across its middle part.

Table 1 Comparison between electromagnetic flow measurements and doppler velocity measurements for individual heart beats in one measurement series

The comparison was performed on the common carotid artery for every third heartbeat. The table illustrates the typical degree of scatter between different cardiac cycles in a steady state situation.

| Observation | EM flow (ml/min) | Scale factor | Doppler flow (ml/min) | Calculated radius (mm) |
|-------------|------------------|--------------|-----------------------|------------------------|
| 1 | 219.1 | 13.7 | 210.3 | 1.45 |
| 2 | 216.3 | 13.8 | 210.1 | 1.46 |
| 3 | 216.3 | 13.9 | 211.6 | 1.46 |
| 4 | 234.3 | 14.1 | 234.7 | 1.47 |
| 5 | 235.3 | 14.3 | 228.6 | 1.48 |
| 6 | 232.3 | 14.3 | 223.1 | 1.48 |
| 7 | 219.2 | 14.0 | 216.0 | 1.47 |
| 8 | 214.7 | 14.0 | 211.9 | 1.47 |
| 9 | 216.6 | 14.1 | 208.2 | 1.47 |
| 10 | 199.9 | 13.9 | 190.4 | 1.46 |
| 11 | 195.3 | 13.6 | 182.0 | 1.45 |
| 12 | 196.3 | 13.6 | 178.3 | 1.45 |
| 13 | 185.5 | 13.1 | 176.5 | 1.42 |
| 14 | 162.3 | 11.5 | 154.4 | 1.33 |
| 15 | 197.9 | 13.4 | 186.5 | 1.43 |

Median

Confidence interval with confidence probability 0.95

[1.45-1.47]

Only two transducers have been used in the investigation: one 6 MHz probe and one 1.5 MHz probe. Fig. 1 shows the intensity distribution across the beam for various selected distances from the probe face for these two transducers. The intensity distribution was measured by means of a small ceramic crystal which was moved in different positions relative to the sound probes in a water tank.

The electromagnetic flowmeter

A standard electromagnetic blood flow meter (type 372 Nycotron A/S Norway) Care was taken to ensure a close fit of the transducers around the vessel without compressing it. Since drift of the zero voltage may be a problem with electromagnetic flowmeters this voltage was determined repeatedly during the experiment by total compression of the artery. The transducers were calibrated in an *in vitro* system before and after the experiments.

Comput. procedures

The doppler frequency Δf caused by blood cells moving with velocity v is given by

$$\Delta f = \frac{2f_0 \cos \theta}{c}$$

where f_0 is the emitted sound frequency (in our case either 6 or 1.5 MHz), θ is the angle between the direction of the sound beam and the blood velocity, and c is the velocity of sound in the tissue (approximately 1500 m/s). This formula is of course equally true if both Δf and v are substituted by the corresponding mean doppler frequency $\bar{\Delta f}$ and mean blood velocity \bar{v} across the vessel for a given time interval. In addition the instantaneous blood flow Q is given by

$$Q = A \bar{v}$$

Stimulation of gastric acid secretion and suppression of VIP-like immunoreactivity by bombesin in the Atlantic codfish *Gadus morhua*

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HOLSTEIN B. & HUMPHREY C.S., Stimulation of gastric acid secretion and suppression of VIP-like immunoreactivity by bombesin in the Atlantic codfish, *Gadus morhua*. Acta Physiol Scand 1980, 109: 217-223. Received 26 Oct. 1979. ISSN 0001-6772. Department of Zoophysiology, University of Göteborg, Göteborg, Sweden and Department of Surgery, Massachusetts General Hospital, Boston, Mass. 02114, USA.

Cods were equipped with cannulas for drainage of the stomach and for separate perfusion of the stomach (pure sea-water) and intestine (diluted sea-water). Acidity and volume of gastric effluence were measured. Plasma immunoreactive gastrin and vasoactive intestinal polypeptide (VIP) were assayed in some experiments. The high rate of "basal" acid secretion was further elevated by intravenous administration of bombesin, but not by pentagastrin. Exogenous VIP inhibited acid secretion. Following 5 h of bombesin infusion, plasma gastrin-IR was unaffected while VIP-IR was depressed compared to saline-treated controls. The possibility that bombesin stimulates acid secretion by inhibiting VIP-release is discussed.

Key words: Bombesin, gastric acid secretion, gastrin, fish physiology, VIP, radioimmunoassay.

Bombesin, a tetradecapeptide isolated from frogs (Anastasi et al 1971) stimulates gastric acid secretion in man, cat and dog (Bertaccini et al 1973, 4; Erspamer & Melchiorri 1975), but not in the (Bertaccini et al 1973). The effect is mediated the release of antral gastrin (Basso et al 1974; Bertaccini et al 1974; Impicciatore et al 1973, 14). There is evidence for the presence of bombesin-like material in the gastrointestinal tract of frog, B. and man (Polak et al 1976; Walsh & Holmstedt 1976).

In codfish, rendered water-deficient by pyloric ligation, basal acid secretion is low or absent (Holstein 1979). Histamine and carbachol are effective secretagogues but no response was noted by intravenous pentagastrin (Holstein 1975, 1976) or bombesin (Holstein, unpublished). The finding in cods which have been water-repleted by intrastomal perfusion with diluted sea-water exhibit high rates of "basal" acid secretion (Holstein 1979) called for reinvestigation of pentagastrin and bombesin effects on gastric acid secretion. In a parallel, and intentionally separate investigation,

we examined the possible involvement of vasoactive intestinal polypeptide (VIP) in the regulation of codfish acid secretion. Some results from that work is included in the present report.

MATERIALS AND METHODS

Animals. Codfish of both sexes, weighing 365-620 g were used. Before experiments they were kept in storage aquaria with recirculating, chilled (10°C) sea-water for at least one week. They were not fed.

Surgery. The animals were surgically prepared for the separate perfusion of the stomach and intestine and for drainage of the stomach. The technique has been described in detail (Holstein 1979): the cannulas positioned were C1, C2 and C3.

Postsurgical treatment. Immediately following recovery from anaesthesia (MS-222, Sandoz) the fishes were transferred to smaller tanks and perfusion of the intestine (33% SW) and stomach (100% SW) containing

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100% SW indicates pure sea-water. 33% SW is a mixture of one part sea-water and two parts distilled water.

own right. Our results so far have, however, shown that the scale factors correspond to internal diameters of the right order of magnitude.

We would like to thank Halvor Hobæk, Bergen, for help with the measurements on the ultrasound transducers shown in Fig. 1. Knut Liestøl and Arild Njå for valuable comments on the manuscript. M. T. was supported by a student fellowship from The Norwegian Research Council for Science and the Humanities. Financial support was also provided by The Royal Norwegian Council for Scientific and Industrial Research and by Vingmed A/S.

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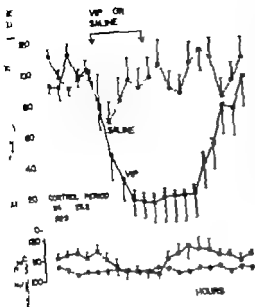


Fig. 3. Effect of saline ($n=8$) and $20 \mu\text{g/kg h}$ VIP ($n=5$) on gastric acid secretion and volume output.

in albumin. This antibody shows no evidence of cross-reactivity with other peptides. The low or limit of detection is 5 pmol . All samples from any one experiment were run together.

Statistics

Results are presented as mean \pm S.E. or as mean and S.E. For comparison of means, the Mann-Whitney U -test or the Kruskal-Wallis one-way analysis of variance (all 1996) were used. The level of significance was set at 0.05 (two-tailed).

RESULTS

Secretory effect

Pentagastrin. The pentapeptide was administered at 2 and $20 \mu\text{g/kg h}$ (Fig. 1). In both series dur-

ing the control period, the secretory rate was increasing. During the 1st treatment h (lower dose) a value significantly different from the control was obtained, and in the late post-treatment period (higher dose) depressed acid secretion was noted. The result presents no convincing evidence that the pentapeptide, at the doses employed, possesses any stimulatory effect.

2. Bombesin. The tetradecapeptide was administered at 0.5 and $2.5 \mu\text{g/kg h}$. Both doses increased the secretory rate (Fig. 2). With the lower dose stimulation was encountered in all the 10 individuals, while with the higher dose 10 were stimulated and 2 inhibited.

3. VIP. $70 \mu\text{g/kg h}$ was administered to 5 animals. Acid secretion rapidly decreased and remained depressed for 7 h (following the onset of the peptide (Fig. 3)).

Plasma gastrin-IR and VIP IR

The effect of 5 h VIP or bombesin administration on plasma levels of gastrin-IR and VIP IR appears in Table 1. The secretory effects confirming the above, are shown in Fig. 4. VIP injected at $20 \mu\text{g/kg h}$ increased the plasma level of VIP IR to about 3 times that of saline-treated controls, and tended to decrease the level of gastrin-IR. The later effect was not statistically significant. Bombesin ($2.5 \mu\text{g/kg h}$) depressed the level of VIP IR but did not affect gastrin-IR. There was no difference (by analysis of variance) among the % b.wt. values (Table 1). These values indicate that the fishes, at the time of sacrifice, were in proper water balance (Holmsten 1979a).

Volume effects

Gastric volume outputs appear in the lower panels of the figures and are expressed as percentages of

Table 1. Final body weights and plasma concentrations of IR-gastrin and IR-VIP in fishes treated with the VIP ($20 \mu\text{g/kg h}$) or bombesin ($2.5 \mu\text{g/kg h}$) during 5 h.

Five animals in each group. Gastric acid secretion is shown in Fig. 4. The P -values are vs. the saline series (Mann-Whitney U -test).

| Treatment | % b.wt. | IR-gastrin (pg/ml) | IR-VIP (pg/ml) |
|-----------|-----------------|-------------------------------|---------------------------------|
| Saline | 101.5 ± 0.5 | 35.4 ± 2.9 | 418.6 ± 57.8 |
| VIP | 101.1 ± 0.5 | 27.6 ± 3.2 ($P=0.15$ NS) | 1366.0 ± 79.7 ($P=0.008$) |
| Bombesin | 102.4 ± 0.6 | 34.8 ± 4.0 ($P=0.84$ NS) | 173.6 ± 30.9 ($P=0.008$) |

% body weight at the time of sacrifice expressed as percentage of the pre-surgical b.wt.

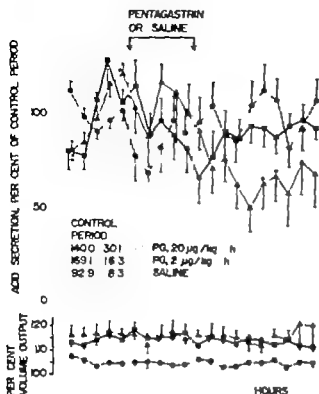


Fig. 1 Effect of saline and two doses of pentagastrin 2 and 20 µg/kg h on gastric acid secretion and gastric volume outflow. The series comprised 8, 10 and 5 animals respectively. In this and the following figures the mean secretory rates (pmol H⁺/kg h \pm S.E.) during the respective control periods are given. Asterisks denote a significant difference ($P < 0.05$, $P < 0.01$) between saline-treated and hormone-treated animals.

100 mg/l phenol red as a volume marker started. Perfusion rate for both stomach and intestine was about 8.5 ml/h but varied between pumps and experiments. The actual rate was recorded following each experiment. Gastric outflow was collected in 1 h fractions. The fishes were left without further treatment for at least 20 h.

Experimental protocol

1. *Secretory effects of exogenous peptides* Pentagastrin (Peptavlon, ICI), bombesin (a generous gift from Dr R. de Castiglione, Farmitalia, Milano), VIP (natural porcine VIP generously supplied by Prof. V. Mutt, GIH Laboratory, Karolinska Institutet, Stockholm) or saline (1.05% NaCl) was injected for 5 h. Gastric acid secretion was assayed for 5 h before during and for 10 h following treatments. All injections were by the i.m. route at a rate of 0.62 ml/h.

2. *Effect of bombesin and VIP on plasma gastrin- and VIP-immunoreactivity* Following 5 h of bombesin, VIP or saline administration the fishes were removed from the aquaria, stunned with a blow on the head and 5 ml of blood drawn from the caudal vein. The syringe contained 5000 KIU aprotinin (Sigma) and 200 IE heparin (Vitrum). The plasma was separated by lyophil red and sent to Boston for peptide analyses. Gastric acid secretion was assayed for 5 h before and during treatments.

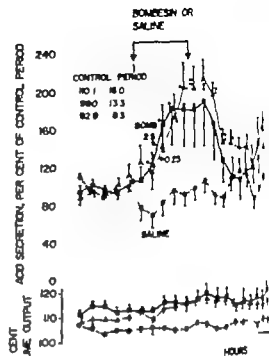


Fig. 2 Effect of saline and two doses of bombesin 2.5 and 0.25 µg/kg h. The series comprised 8, 10 and 5 animals, respectively.

Analyses and presentation of the data

1. *Gastric secretion* The samples were analysed for acidity, volume and phenol red (PR) concentration as described earlier (Holstein 1979a). From the amount of PR infused and the amount of PR in the effluent the recovery was calculated and the outflow was accordingly corrected. The acid content was calculated from acidity and corrected volume and expressed as H⁺/kg h. In calculating this, the presurgical value was used. In the figures, acid secretion has been given as the mean acid secretion during the 5 h (control) preceding change of the experimental situation, i.e. 100%. Gastric volume output is given as a percentage of the hourly infused volume.

2. Radioimmunoassays

1. *VIP* Plasma VIP concentration was measured by a RIA described previously (Ebeli et al. 1978). The antibody raised in rabbits against highly purified VIP conjugated to bovine serum albumin. Highly purified natural and synthetic VIP produced equal displacement of labelled VIP from the antibody. The assay had a limit of detection of 7.5 pg/ml. All samples from one experiment were assayed together in triplicate with determinations of nonspecific binding in the absence of antibody. No cross reactivity has been detected with a number of peptides in concentrations up to 10⁴ times including the related peptides secretin, glucagon and gastrin. Gastrin was assayed in a previously described RIA (Dent et al. 1972) using antibody prepared in rabbits against synthetic human gastrin 17 conjugated to

glucagon-like immunoreactivity is present in same cell (see Falkmer & Östberg 1976). Although the common C-terminal sequence of gastrin CCK appears to be present, this study failed to give evidence that this sequence alone (i.e. pentapeptide) is sufficient for the stimulation of acid secretion in the water-repleted cod. Therefore the failure of pentagastrin to induce acid secretion in water-depleted cods (Holstein 1975) is not solely due to the dehydration. Dehydration could, however, explain the inefficiency of bombesin in water-depleted cods (Holstein, unpublished). The exact mechanism remains to be worked

out. Regulation of acid secretion by bombesin in rats and man and the concomitant release of gastrin (Basso et al. 1974, 1975; Bertaccini et al. 1973, 1974; Impicciatore et al. 1973, 1974), together with the finding of bombesin-like material in several species (Polak et al. 1976, 1978; Walsh & Jansky 1976) suggest that a bombesin-like peptide may play a role in the regulation of gastrin release (Ersparmer & Bleichborn 1975). In concert with this suggestion is the evidence presented by Jansson et al. (1976) and by Llanos et al. (1977) that gastrin release following intestinal feeding may involve an endogenous bombesin-like peptide. In the codfish, too, bombesin stimulated acid secretion but it did not elevate the level of plasma gastrin-IR. Thus, the mechanism appears not to be based on the release of gastrin and therefore is different from that in mammals. The uncertainty involved in this conclusion, due to incompletely characterized antigen (codfish 'gastrin') and antibody used, should be stressed. The conclusion seems never supported by the inefficiency of pentagastrin. Although direct activation by bombesin of acid secretory cell seems excluded in mammals (Jansson et al. 1975; Impicciatore et al. 1973), such a mechanism cannot be excluded in the codfish. However it is also possible that bombesin releases a stimulatory principle not detected by the gastrin assay. Such a mechanism was suggested by Hirs & Ozols (1978) who wrote: "Bombesin is a potent gastric stimulus whose action is only partially explained by the measured gastrin release. Release of CCK by bombesin has been suggested (Konturek et al. 1976b) and there is evidence for release also of pancreatic polypeptide" (Taylor et al. 1977) and both peptides stimulate mammalian acid secretion in the absence of

gastrin (Grossman 1971; Lin et al. 1977). The octapeptide of CCK inhibits 'basal' secretion in the cod (Holstein, unpublished) making CCK less attractive as the stimulatory principle and PP has not been tested for secretory effects in fish, though its presence in several teleost species has been demonstrated (Van Noorden & Parent 1978).

Since the secretion of acid reflects the sum of stimulatory and inhibitory agents impinging on the secretory cell, there is also the third possibility that bombesin increases acid secretion by suppression of an inhibitor. We found a significant decrease in plasma VIP-IR and therefore, concerning the cod, consider the third alternative likely. The increase in a gastrin-like peptide not detected by the gastrin assay can, however, not be excluded. The suggestion that bombesin stimulates acid secretion by inhibition of VIP release is tempting, but no significant correlation was found between plasma VIP-IR levels and acid secretion (in absolute terms or as a per cent of control period) in the bombesin-treated animals. The importance of this, however, is difficult to evaluate as long as the individual components driving the 'basal' secretion are qualitatively and quantitatively unknown. Pertinent is the observation that feeding results in an initial inhibition of plasma VIP-IR in the dogfish shark (Humphrey unpublished).

Volume output. Theoretically gastric volume output includes (i) infused volume (ii) ingested volume and (iii) gastric volume secretion. Perfusion of the intestine, with pure or diluted sea-water at the rate previously used, prevents drinking (Holstein 1979, 1979b). During intestinal acidification, however, drinking occurs and mean volume output amounts to above 200% (Holstein 1979b). Also intestinal perfusion with isosmotic glucose (Holstein & Cederberg 1980) increases volume output (to a mean of 160%)—this increase probably also being an effect of oral water ingestion. The volume changes encountered in the present experiments were of much less magnitude and most likely reflect changes in gastric volume secretion. The most obvious deviation from the covariation between acid secretory rate and volume output occurs in the VIP postinjection period (Fig. 3), and could indicate that acid secretion can change independently from volume secretion. Effects on water ingestion seem unlikely but are not excluded and may contribute.

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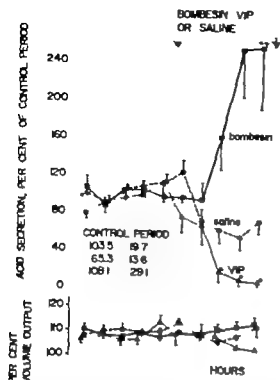


Fig. 4. Effect of saline, VIP ($20 \mu\text{g/kg h}$) and bombesin ($2.5 \mu\text{g/kg h}$). 5 animals in each group. Following the 5th treatment h blood samples for hormone assay were collected (Table 1).

the gastric perfusion rate. Despite this normalization the outputs varied between the different series and were therefore not statistically compared. Generally the change in volume output mirrors the change in acid secretory rate. Thus no obvious change was noted during pentagastrin infusion (Fig. 1) while during VIP administration volume output decreased (Figs 3, 4). However following termination of VIP volume output recovered more rapidly than acid secretion (Fig. 3). Bombesin which stimulated acid secretion increased volume output (Figs 2, 4) the increase being most obvious during the lower dose.

DISCUSSION

Acid secretion. VIP is a polypeptide of 28 amino acids isolated from hog small intestine (Said & Mutt 1970) has a wide spectrum of biological actions (Said 1975). Mammalian gastric acid secretion stimulated by food (Villar et al. 1975, 1976), histamine (Konturek et al. 1976a), pentagastrin (Makhoul et al. 1978; Schorr et al. 1974) and gastrin (Villar et al. 1975) is inhibited while basal secretion is not (Villar et al. 1976). VIP also inhibits stimu-

lated gastrin release but does not affect levels of gastrin (Villar et al. 1975). It has been suggested that blockade of gastrin at the parietal cell level is the major mechanism by which VIP inhibits gastric acid secretion (Konturek et al. 1976a). The present results indicate that a VIP-like molecule circulates in the codfish. The basal plasma concentrations are similar to those measured in dogfish shark (Humphrey unpublished) but when compared to man, dog, and rat ($<1.0 \text{ pg}$ with this assay). Previously by Grossman and Elvidge evidence has been presented for the occurrence of similar material in extracts from cod and pig testis (Dockray 1974, 1975, 1976). The present work also seems to indicate that "basal" acid secretion is depressed by VIP but the nature of the secretion though arbitrarily designated "basal" is incompletely characterized. The secretion is dependent on an intact vagal supply (Holstein & Linderberg 1980) but this does not exclude the involvement of a stimulating hormone. Gastrin was in fact detected in codfish plasma but failure of pentagastrin to stimulate the acid secretion makes it difficult to ascribe part of the "basal" secretion to this immunoreactivity. The plasma level was not significantly affected by even high VIP in an amount elevating the plasma VIP to about 300% of the control level (Table 1). This is in accordance with VIP effects on basal gastric secretion in the dog (Villar et al. 1976). The findings must however be carefully interpreted because (i) the sequence of the gastrin molecule recognized by the antibody used is undetermined and (ii) the "cod gastrin" is not wholly characterized (Larsson & Rehfeld 1977). The mechanism of action of VIP on the cod, whether on the oxynticopeptic cells or on the release of a stimulating peptide is not undetermined.

Using an antibody specific for the common terminus of gastrin and CCK, Larsson & Rehfeld (1977) found endocrine cells in the gut of most of all vertebrate classes including the cod. Antiserum directed against the NH_2 -terminal part of gastrin demonstrated endocrine cells in the fish vertebrates but failed to do so in frog and cat. In these later animals the cells reactive with COOH terminus specific antibody also reacted with an antiserum specific for the sequence 31-41 CCK 33. The authors questioned the existence of the lower vertebrates of gastrin and CCK as separate hormones. (In cyclo tomus gastrin/CCK).

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the effect of insulin on skeletal muscle contractions and its relation to the effect produced by β -adrenoceptor stimulation

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HOLMBERG E. & WALDECK, B. The effect of insulin on skeletal muscle contractions and its relation to the effect produced by β -adrenoceptor stimulation. *Acta Physiol Scand* 1980 109: 225-229. Received 26 Oct. 1979. ISSN 0001-6772. Research and Development Laboratories, AB Draco, Lund, Sweden.

The soleus, a slow-contracting, and the extensor digitorum longus (EDL), a fast-contracting muscle, from the guinea-pig were prepared for measurement of isometric contractions *in vitro*. Insulin, 2.5-55 mU/ml caused dose-dependent depression of twitches and submaximal concentrations of the soleus muscle similar to and additive with that produced by the β_2 -adrenoceptor agonist, terbutaline. The effect of terbutaline but not that of insulin was blocked by propranolol. Insulin had no apparent effect on the contractions of the EDL, whereas terbutaline increased the force of contraction. When depressed by KCl, however, insulin partially restored the twitch tension in both muscles. The possible role of effects on the Na⁺-K⁺ transport is discussed.

Key words. Insulin, β -adrenoceptor, terbutaline, skeletal muscle, contraction, guinea-pig.

Many drugs which affect skeletal muscle contraction. The β -adrenoceptor agonists have received great interest because they have opposite actions: slow- as compared with fast-contracting muscles. In slow-contracting muscles the tension and duration of twitch are decreased while fast-contracting muscles respond with an increase in all parameters (Bowman & Zaiman 1958; Bowman & Nott 1969).

It has been suggested that these functional effects of β -adrenoceptor stimulation are due to changes in intracellular Ca²⁺ fluxes mediated via cAMP (Bowman & Nott 1969, 1974). However, stimulation of β -adrenoceptors in skeletal muscles also increases the activity of the Na⁺-K⁺ transport mechanism of the cell membrane, apparently via cAMP (Clausen & Flatman 1977; Rogers, Cheng & Herlihy 1977).

To test the possibility of a causal relationship between the stimulation of the Na⁺-K⁺ transport and the changes in contraction we have studied the effect of insulin. This hormone has long been known to affect ionic fluxes in skeletal muscle tissue (1964; Zierler 1966). More recent studies

show that the active transport of Na⁺ and K⁺ ions through the cell membrane of the muscle is stimulated (Clausen & Kohn 1977) presumably by a mechanism unrelated to changes in cAMP (Flatman & Clausen 1979b).

Further, we have compared the effect of insulin on skeletal muscle contractions with that of terbutaline, a β_2 -selective adrenoceptor agonist (Bergman, Persson & Wetterlin 1969; Persson & Olsson 1970). Measurements have been made on isolated preparations of the soleus, a slow-contracting and the extensor digitorum longus (EDL), a fast-contracting muscle from the guinea-pig (Ariano, Armstrong & Edgerton 1973).

METHODS

Male guinea-pigs, about 200 g, were used. The soleus and the EDL muscles were dissected out under pentobarbital anaesthesia and were mounted in organ baths containing oxygenated Krebs solution at 37°. Submaximal contractions were evoked by transverse field stimulation. Trains of supramaximal pulses of 0.5 ms duration were delivered every 20 s from Grass S 88 stimulator. For

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METHODS

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Male guinea-pigs, about 200 g, were used. The soleus and the EDL muscles were dissected out under pentobarbitone anaesthesia and were mounted in organ baths containing oxygenated Krebs solution at 37°. Submaximal contractions were evoked by transverse field stimulation. Trains of supramaximal pulses of 0.5 ms duration were delivered every 20 s from Grass S 88 stimulator. For

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Fig. 1 The effects of insulin and terbutaline on the force of contraction of an isolated soleus muscle from guinea pig. Subtetic contractions and twitches were measured alternately. Terbutaline $2.3 \mu\text{mol/l}$ was added at the left arrow (TRB). After rinsing and recovery insulin 50 mU/ml was added (INS) the right arrow.

the soleus the stimulus frequency was about 12 Hz and the train duration 1.5 s. For the EDL these parameters were 40 Hz and 150 ms, respectively. Ten seconds after each train a single pulse was given to monitor the twitch tension of the muscles. All measurements were made isometrically. For more details see Waldeck (1976).

When stable contractions had been established the response to a supra-maximum dose of terbutaline ($2.3 \mu\text{mol/l}$) was determined. The mean maximum depression \pm S.D. of the soleus muscle was $32 \pm 6\%$. The subtetic contractions of the EDL muscles increased by $56 \pm 18\%$. After a rinsing and recovery period the muscles were ready for use. Details of the experiments are given in the results section.

The following drugs were used: Porcine insulin (Sigma), terbutaline sulphate (Draco) and propranolol (ICI). Solutions were made up in saline. Statistical evaluation of the data was made by Student's *t*-test.

RESULTS

(a) Pilot studies

Insulin caused in the soleus muscle a depression of subtetic contractions, qualitatively similar to that produced by terbutaline (Fig. 1). However the effect of insulin developed more slowly than that of terbutaline and the maximum effect appeared to be smaller. In the EDL insulin up to 50 mU/ml had no

Table 1 Effect of insulin on the force of subtetic contractions and twitches of the isolated soleus muscle from the guinea-pig

The data are the means \pm S.E. of 5 expts

| Insulin (mU/ml) | Per cent depression | |
|--------------------|--------------------------|----------------|
| | Subtetic contractions | Twitches |
| 2.5 | 7.6 ± 1.7 | 4.6 ± 1.6 |
| 12 | 11.0 ± 3.0 | 7.4 ± 2.3 |
| 55 | 14.8 ± 2.0 | 10.8 ± 2.9 |

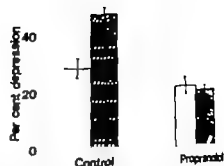


Fig. 2 Combined effect of insulin and terbutaline and inhibition by propranolol on subtetic contractions of isolated preparations of the guinea-pig soleus muscle. The open columns show the effect of 25 mU/ml insulin. The hatched columns show the effect after a further addition of $2.3 \mu\text{mol/l}$ terbutaline. When present, propranolol $0.3 \mu\text{mol/l}$ was added 30 min before insulin. Mean \pm S.E. of 5 expts.

effect on the force of contraction but a slight depression which could not be distinguished from the usual slow deterioration of the preparation (Holmberg & Waldeck 1977).

In our pilot studies we also found that once a muscle had been exposed to insulin it was sensitive to further additions of the hormone, even after washing. Therefore each muscle was used only once in the following experiments.

(b) Dose response relation for insulin

In the soleus muscle insulin from 2.5 to 55 mU/ml caused a dose-dependent depression of the subtetic contractions as well as of the twitch tension ($P < 0.05$). The effect on the subtetic contractions tended to be greater than that on the twitches (Table 1).

The mean time \pm S.E. to reach half the effect produced by 55 mU/ml insulin was in 5 expts $4.8 \pm 0.3 \text{ min}$. The corresponding figure for the maximum effect of terbutaline is of the order 1 min (Holmberg, Svedinger & Waldeck 1979).

(c) The effect of propranolol

In the next experiment on soleus muscles insulin 25 mU/ml was given and after about 15 min when the effect had developed terbutaline $2.3 \mu\text{mol/l}$ was added to the bath. In half of the expts propranolol $0.3 \mu\text{mol/l}$ had been given 30 min before insulin. The result is shown in Fig. 2.

Insulin caused a depression of the force of subtetic contractions by 20–30%. Terbutaline caused a further depression of the contractions.

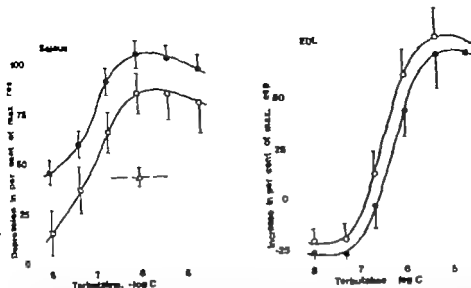


Fig. 3. Cumulative dose-response curves for terbutaline in the presence and in the absence of insulin. Insulin, 25 mU/ml, given to preparations of soleus and EDL muscles. After about 30 min. terbutaline was added cumulatively (closed circles). Control muscles without insulin were run in parallel (open circles). The effect of insulin alone on the soleus is denoted by a triangle. Shown are the means \pm S.E. of 7-8 expts. Ordinate: Changes in the force of subtetanic contractions in per cent of the maximum response to a test dose of terbutaline (see Methods).

it being almost doubled ($P < 0.005$). Propranolol did not affect the insulin-induced depression but blunted completely that caused by terbutaline.

Interaction between insulin and terbutaline

Insulin, 25 mU/ml was given to preparations of soleus and EDL muscles. After about 30 min, when effect of insulin had developed, terbutaline was added cumulatively. Control muscles without insulin were run in parallel. The effects were calculated initially in per cent of the maximum response to $1 \mu\text{mol/l}$ of terbutaline. The changes refer to the time at the time for the addition of insulin.

In the previous expts. insulin caused a depression of the subtetanic contractions of the soleus muscle (Fig. 3). Terbutaline added to this effect in a dose-dependent manner and at $0.22 \mu\text{mol/l}$ the blunted effect was twice that elicited by insulin alone ($P < 0.001$) but less than that produced by terbutaline at that dose level ($P < 0.05$). Moreover, the maximum depression obtained by terbutaline appeared to be greater in the presence than in the absence of insulin, but this difference did not become statistically significant.

There was no apparent effect of insulin on the contractions of the EDL muscle. Nor did insulin change the response to terbutaline in this preparation (Fig. 3). However, during the time for the

preincubation with insulin, both the treated and the control muscles lost in force some 10% (cf. Holmberg & Waldeck 1977). This is the reason why the cumulative dose-response curves for terbutaline start at a point below zero.

(e) Effect of insulin on muscle depressed by KCl

In some experiments on soleus and EDL muscles KCl was added to the bath until the contractions of the muscles were depressed by about 50%. This occurred at a final $[K^+]$ concentration of 12-13 mmol/l, usually higher for the EDL than for the soleus. Then insulin, 25 mU/ml was added.

In all expts. the progress of the depression caused by excess K^+ was broken and reversed by insulin in a few minutes. The increase in the force of contraction calculated in per cent from the bottom of the dip ranged from 9-53% in 5 expts. on the soleus. In 4 expts. on the EDL the increase ranged from 15-41%. The increase in the force of contraction of the EDL was significantly different from zero ($P < 0.05$). Records from 2 of the expts. are shown in Fig. 4.

DISCUSSION

Insulin caused a dose-dependent depression of the subtetanic contractions of the soleus muscle similar

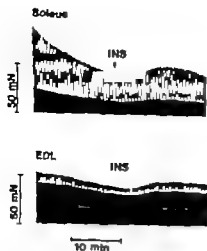


Fig. 4. Partial reversal by insulin of the depression of the force of contraction caused by excess K^+ in the medium. Subtetanic contractions and twitches were measured alternately. The $[K^+]$ was 13 and 14 mmol/l for the soleus and the EDL, respectively. The addition of 25 mU/ml insulin is indicated by an arrow.

to that produced by terbutaline. It is pertinent that this effect occurred in the same dose range as the stimulation by insulin of the Na^+ - K^+ transport in the rat soleus *in vitro* (Clausen & Kohn 1977). The failure of propranolol to inhibit the effect of insulin indicates that this hormone acts on a site beyond the β -adrenoceptor.

In a previous paper (Holmberg & Waldeck 1980) we have pointed out the similarity between the dose response curves for the effect of β -adrenoceptor agonists on the force of contraction (Waldeck 1977) and those on the Na^+ - K^+ transport (Clausen & Flatman 1977) in the soleus muscle. Further the effects of insulin and terbutaline on subtetanic contractions are additive (the present data) as are the effects of insulin and adrenaline on the Na^+ - K^+ transport (Flatman & Clausen 1979a and b). Moreover the observation that the effects of insulin on the Na^+ - K^+ transport are less pronounced and slower in onset than those of adrenaline (Flatman & Clausen 1979a and b) also parallels our functional studies on insulin and terbutaline.

These data suggest that there is a causal relationship between the stimulation of the Na^+ - K^+ pump of the cell membrane and the depression of the contractions of the soleus muscle. The stimulation by β -adrenoceptor agonists and insulin of the Na^+ - K^+ transport in the soleus muscle is blocked by ouabain (Clausen & Flatman 1977; Clausen & Kohn 1977). Experiments to block by ouabain the effects of the agents on the force of contraction are

more difficult to interpret because ouabain also affects the contractile force of the muscle (Tashiro 1973; Holmberg & Waldeck 1980 and unpublished data). However the view that changes in the Na^+ balance may control the intracellular concentrations of Ca^{2+} which regulate the contractile machinery of the muscles is supported by recent evidence (Tashiro 1973; Bowman 1980; Holmberg & Waldeck 1980).

Insulin had no detectable effect on the force of contraction of the EDL under normal conditions. Moreover the twitch tension of the soleus muscle was decreased after insulin with no significant secondary increase as observed after terbutaline (Holmberg & Waldeck 1980). It thus appears that insulin mimics the twitch-depressing but not the twitch-enhancing effect of β -adrenoceptor agonists on skeletal muscle contractions. One possible explanation of this difference is that the major factor in the terminal distensibility of the sarcolemma is the terminal cisternae of the sarcoplasmic reticulum resulting in increased release of Ca^{2+} . The insulin molecule, due to its size, would not enter sites in the interior of the T-tubes whereas the adrenoceptor agonists do, although after a delay (cf. Holmberg, Svedinger & Waldeck 1979). However alternative explanations have to be left open.

Like terbutaline (Holmberg & Waldeck 1980) insulin partly restored the twitch tension of both soleus and the EDL muscles when depressed by KCl . A similar observation has been made by Bowman & Raper (1964) in experiments with reserpine and adrenaline on the KCl -depressed rat diaphragm. This effect also may be due to the ability of insulin to stimulate the transport of Na^+ and K^+ across the cell membrane and thus cause repolarization. An alternative explanation of the twitch potentiation by insulin is that it is related to extracellular glucose transport (Bowman & Raper 1964) but our data indicate that the effect of insulin on the Na^+ - K^+ transport is distinct from those on the transport of sugars and amino acids (Clausen & Kohn 1977) and the role of glucose transport is still unclear.

In our experiments with insulin we observed tachyphylaxis to the effect of the hormone. This phenomenon has been reported also by others (Bowman & Raper 1964). Therefore experiments with insulin have to be carefully standardized in the hormonal state of the animals at the beginning of the experiment may influence the outcome (Brodie et al. 1974).

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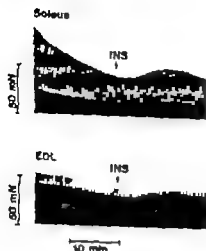


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mulation of HCO_3^- transport by gastric inhibitory peptide P) in proximal duodenum of the bullfrog

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Proximal duodenum isolated from the bullfrog actively transports HCO_3^- into the lumen, a process which is inhibited by acetazolamide and nifedipine and stimulated by prostaglandins (Flemström 1979, 1980a). Duodenal mucosa in this animal is devoid of Brunner glands, indicating that HCO_3^- transport is a property of the surface epithelial cells. HCO_3^- is secreted by duodenal epithelium also in the dog *in vivo* and acetazolamide raises the rate of loss of intraduodenally testis-steroid in this species (Harmon, Woods & Garfield). These results suggest that epithelial HCO_3^- transport contributes to duodenal disposal of luminal acid. The ability of gastric inhibitory peptide (GIP) to inhibit gastric emptying and acid secretion (Polak & Bloom 1977) should decrease delivery of H^+ ions into the duodenum. The latter may indicate of an ulceroprotective function and it is of interest to examine the effects of GIP on duodenal epithelial HCO_3^- transport.

Methods. External muscle layers were removed from the proximal duodenum of the bullfrog (*Rana temporaria*) and the resulting mucosal cylinder, 12 mm long, mounted between two glass coverslips connected to a reservoir. An unbuffered bathing solution (5 ml) was circulated by a perfusion pump and HCO_3^- transport stimulated at pH 7.40 with 4 mM HCl under control from a pH system (Radiometer, Copenhagen, Denmark). Serosal (serosal) side solution (250 ml) was buffered at pH 7.20 with 0.1 M phosphate and 8 mM HCO_3^- and contained glucose (2 mM). This solution was gassed with mixture of 95% O_2 and 5% CO_2 . Both solutions contained Na 102.4 K, Ca 1.8, Mg 0.8 and Cl 91.4 mM. Mannitol 150 were added to the luminal solution to give isotonicity (221.8 mosM). GIP was added to serosal side in small volume (50 μl for 10^{-8} M). Trans-epithelial electrical potential difference was

measured in two matched calomel electrodes. Effects of GIP on HCO_3^- transport were examined also in gastric fundic mucosa from *Rana temporaria* in which H^+ secretion was abolished by pretreatment with 10^{-4} M Metiamide (Flemström 1977). Compositions of the bathing solutions and titration of HCO_3^- transport were as described for duodenum. Purified porcine GIP was a kind gift from Professor Viktor Mutt, Department of Biochemistry II, Karolinska Institutet, Stockholm, Sweden.

Results. In untreated duodenum there was a small continual rise in the electrical potential difference and a tendency for the rate of HCO_3^- transport to decline over a period of 4 h (Fig. 1). Administration of GIP (10^{-8} M) caused a significant increase ($0.01 < P < 0.02$) in the rate of HCO_3^- transport compared with that in the preceding control period. Addition of 10^{-8} M GIP resulted in a further increase ($P < 0.01$) compared with the rate at 10^{-10} M and in 3 expts. a still further rise of 0.05–0.10 $\mu\text{eq/h cm}^2$ occurred when the concentration was increased to 10^{-6} M. The slight variations in transepithelial electrical potential difference in GIP treated duodenum were similar to those observed in untreated controls. In Metiamide-treated fundic mucosae HCO_3^- transport decreased from 0.29 ± 0.03 to 0.20 ± 0.03 $\mu\text{eq/h cm}^2$ during 90 min exposure to 10^{-8} M GIP and to 0.16 ± 0.03 $\mu\text{eq/h cm}^2$ during a further 90 min exposure to 10^{-6} M GIP (means \pm S.E., $n=7$). These changes were somewhat greater than the slight decline in HCO_3^- rate occurring in control mucosae.

Discussion. The present results demonstrate that duodenal surface epithelial transport of HCO_3^- is stimulated not only by prostaglandins with ulceroprotective properties but also by the hormone GIP. A significant increase in transport rate occurred with 10^{-8} M GIP while in this same duodenal preparation, the minimal effective concentrations of

Effects of castration and testosterone substitution on body composition and muscle metabolism in rats

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KROTKIEWSKI J G KRAL J G & KARLSSON J Effects of castration and testosterone substitution on body composition and muscle metabolism in rats. *Acta Physiol Scand* 1980 109 233-237 Received 20 Sept. 1979 ISSN 0001-6772. Department of Rehabilitation Medicine II Department of Surgery II Sahlgrenska Hospital University of Göteborg, Sweden and GIIH Stockholm Sweden

Male weanling Wistar rats were castrated or sham-operated and followed for 12 weeks without substitution or with large (2 mg 14 days) or small (0.2 mg 14 days) intramuscular dose of testosterone enanthate. Castration without substitution was associated with lower body weight and smaller fat cell sizes in different adipose tissue depots. The epididymal and caudal subcutaneous depots were the most sensitive to castration. The percentage of fast-twitch high-oxidative (type II A) muscle fibers decreased in the non-substituted castrated animals. There was decrease in phosphorylase and lactate dehydrogenase activities in the white portion of the gastrocnemius muscle of the castrates. These changes were reversed by the large dose of testosterone. Removal of testosterone by castration thus seems to "feminize" male rats with respect to body composition and muscle metabolism.

Key words: Castration, testosterone, body composition, fat cells, muscle fibers, muscle enzymes.

♂ species males have a large muscle mass and a relatively greater strength than females. This difference between sexes is not apparent until puberty at which time changes occur in work performance and muscle metabolism (Enlissou 1973). Differences in the percentage distribution and the area of muscle fibers have been found in female (Gutman et al 1969) and male (Nygaard et al 1969; Edstrom et al 1969). The findings have been attributed to depend on differences in physical activity rather than on hormonal effects (Saltin et al 1971). Since puberty is characterized by increased secretion of gonadal hormones it seems plausible that the sex differences in body composition and muscle metabolism are being due to these hormones. The aim of this study was to elucidate the effects of castration and substitution of testosterone on body weight and composition and skeletal muscle composition and enzyme activities in young male rats.

♂ rats were maintained in temperature-controlled rooms (21°C) with stable humidity (50-60%), fed ad libitum with commercially available rat pellet diet (EWOS Sölertälje Sweden) and had free access to tap water.

The rats were randomly assigned to 4 groups of 10 rats (I-IV). After being weighed 3 of the groups (I-III) were castrated by simple orchectomy, sparing the epididymis and its fat pad (Kral & Thiel 1976), while one group (IV) was sham-operated using the same scrotal incision and handling the testes to simulate the operative trauma. The operations were performed in pentobarbital anesthesia (Mebumal ACO Sweden). Because of pulmonary infection one rat was excluded from the sham-operated group IV.

The rats were allowed to recover for one week before starting the series of bi-weekly intramuscular injections of testosterone enanthate (Testoviron-Depot Schering AG Berlin W Germany), or only the same volume of the vehicle squalen oil according to the following plan:

Castrated rat

Group I: large dose: 2 mg of testosterone every 14 days
Group II: small dose: 0.2 mg of testosterone every 14 days
Group III: sham injection: 0.1 ml of squalen oil every 14 days

Sham-operated rat

Group IV: sham injection: 0.1 ml of squalen oil every 14 days

male Wistar SPT rats with mean age

groups in Macrolon

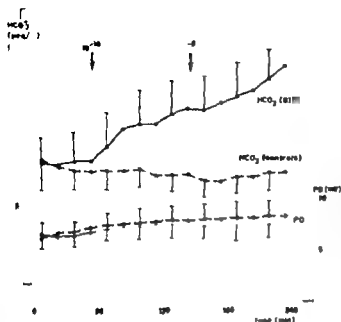


Fig. 1 GIP was added as indicated to the nutrient (serosal) side of isolated duodenal mucosae. Means \pm S.E. of the rate of HCO_3^- transport and trans epithelial electrical potential difference (PD) in GIP-treated mucosae ($n=7$) and untreated controls ($n=9$) are shown.

E type prostaglandins was 10^{-6} M (Flemström 1980a). However, the rate of HCO_3^- transport rose more slowly after GIP compared with the prostaglandins and the maximal rate appears somewhat lower. This may reflect different modes of action and/or slower penetration of the polypeptide from the nutrient solution to HCO_3^- transporting cells in the mucosa.

Gastric mucosa in vitro also displays an active HCO_3^- transport and secretion of HCO_3^- has been demonstrated in guinea pig and dog stomach in vivo. The properties of surface epithelial transport of HCO_3^- in stomach and duodenum are similar but not identical (Flemström 1980b). Evidence obtained in the dog suggests that gastric HCO_3^- transport is controlled by a humoral mechanism since acid is still secreted into the main stomach stimulated HCO_3^- secretion from a denervated Heidenhain pouch (cf

Allen & Garner 1980). The inhibitory action of GIP on gastric HCO_3^- transport in vitro makes unlikely that this hormone is involved in humoral stimulation of HCO_3^- secretion in the stomach in vivo. Humoral control of duodenal surface epithelial HCO_3^- transport in vivo has not been studied and further work is required to evaluate the possible function of GIP as a mediator of duodenal protection. The abnormally high serum concentration of GIP in some patients with duodenal ulceration (Cataland et al 1977) may possibly reflect decreased feedback inhibition of hormone release.

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Effects of castration and testosterone substitution on body composition and muscle metabolism in rats

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Male growing Wistar rats were castrated or sham-operated and followed for 1 week without substitution or with large (2 mg, 14 days) or small (0.1 mg, 14 days) intramuscular dose of testosterone enanthate. Castration without substitution was associated with lower body weight and smaller fat cell sizes in different adipose tissue depots. The epididymal and caudal subcutaneous depot were the most sensitive to castration. The percentage of fast-twitch high-oxidative (type IIA) muscle fibers decreased in the non-substituted castrated animals. There was decrease in phosphorylase and lactate dehydrogenase activities in the whole portion of the gastrocnemius muscle of the castrates. These changes were reversed by the large dose of testosterone. Removal of testosterone by castration thus seems to 'feminize' male rats with respect to body composition and muscle metabolism.

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Most species males have a large muscle mass and consequently greater strength than females. This difference between sexes is not apparent until puberty at which time changes occur in work performance and muscle metabolism (Eriksson 1973). Differences in the percentage distribution and size area of muscle fibers have been found in rats (Gustafson et al. 1969) and man (Nygaard et al. 1969, Edström et al. 1969). The findings have been thought to depend on differences in physical activity rather than on hormonal effect (Saltin et al. 1971). Since puberty is characterized by increased release of gonadal hormones, it seems plausible to explain the sex differences in body composition and muscle metabolism as being due to these hormones. The aim of this study was to elucidate the effects of castration and substitution of testosterone on body weight and composition and skeletal muscle composition and enzyme activities in growing male rats.

METHODS

Forty male Wistar SPF rats with mean body weight 140 g were kept in groups in Macrolon

cages for one week. They were maintained in temperature controlled room (21°C) with stable humidity (50-60%), fed ad libitum with commercially available rat pellet diet (EWOS Södertälje, Sweden) and had free access to tap water.

The rats were randomly assigned to 4 groups of 10 rats (I-IV). After being weighed, 3 of the groups (I-III) were castrated by simple orchectomy sparing the epididymis and the fat pad (Kral & Theil 1976) while one group (IV) was sham operated using the same scrotal incision and handling the testes to simulate the operative trauma. The operations were performed in pentobarbital anesthesia (Mebumal ACO, Sweden). Because of pulmonary infection one rat was excluded from the sham operated group IV.

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Table 1

| Group | Mean weight in g (\pm S.D.) | Analysis of variance | |
|--|--------------------------------------|-------------------------|--------|
| | | Groups | P |
| I Castrated—large doses of testosterone ($n=10$) | | I-II | <0.001 |
| Initial weight | 96.9 \pm 5.4 | I-III | <0.001 |
| Final weight | 349.3 \pm 16.8 | I-IV | n.s. |
| II Castrated—small doses of testosterone ($n=10$) | | | |
| Initial weight | 96.5 \pm 6.5 | II-III | <0.001 |
| Final weight | 301.1 \pm 16.4 | II-IV | <0.001 |
| III Castrated—sesam oil ($n=10$) | | | |
| Initial weight | 93.9 \pm 4 | III-IV | <0.001 |
| Final weight | 304.1 \pm 12.7 | | |
| IV Shamop—sesam oil ($n=9$) | | | |
| Initial weight | 96.4 \pm 2 | | |
| Final weight | 350.0 \pm 19.3 | | |

The size of the larger dose calculated from recent data on testosterone secretion rates in rats (de Jong et al. 1973; Free et al. 1973) was chosen to simulate physiological levels of this hormone. All rats were weighed once a week and the food reservoir three times a week to evaluate food intake. After 12 weeks the animals were fasted over-night, weighed and sacrificed by carotid exsanguination in a well-defined pentobarbital narcosis (50 mg/kg) (Arai 1974). Fat depots from different anatomical regions were removed according to procedures previously described by Krokowski & Björntorp (1975), weighed and analysed for determination of fat cell size according to Björntorp et al. (1971).

Muscle tissue. Immediately after sacrifice, before dissection of fat depots, two specimens each of the white part of the gastrocnemius and the soleus muscle were dissected. Access to the soleus muscle was gained through a sagittal incision from the most distal part of the Achilles tendon up to the level of the knee. After division of the tendon the gastrocnemius was dissected free from the soleus thus uncovering the intermuscular fat pad which was removed. Subsequently the soleus was prepared by trimming off excess fat and fascia before dividing it into two equal samples.

The white portion of the gastrocnemius was localized at the level of the knee after incision of the skin. Excess subcutaneous fat and fascia was trimmed off before excision of muscle tissue. Macroscopically this muscle was homogeneously white in appearance in contrast to the dark red color of the soleus. Microscopy was not performed at this stage.

The samples were frozen directly in liquid nitrogen and stored at -80°C until analysed for determination of enzyme activity and muscle fiber distribution. One portion of the muscle specimen was cut in a cryostat at -20°C and stained for myofibrillar ATPase after preincubation at pH 10.3 for classification of type I (low) and type II (fast) fibres. For classification of the subgroups type IIA (fast

twitch high oxidative FTH) and type IIB (fast twitch low oxidative FTL) both NADPH staining (Nicolle 1970) and ATPase staining with preincubation at pH 10.3 was used (Brooke & Haber 1970).

After homogenization and weighing in 200 μl portion of the sample was further diluted in buffer before 70 μl aliquots were added to 1 ml of test medium. No enzyme activity determinations were performed at fluorometric techniques using a Farrand fluorometer² (Farrand Optical Co. New York). The reactions catalyzed by the enzymes under investigation were coupled to NAD/NADH linked reactions, and the change in NADH or NADPH were monitored according to principles outlined by Lowry (1977).

The measurements were made under V_{max} conditions with an excess of substrate. By running standard curves with known amounts of fluorescence the experimental readings could be quantified.

The enzymes investigated in the present study were Mg²⁺ stimulated ATPase (E.C.3.6.1.4) (Thomson 1976), lactate dehydrogenase (E.C.1.1.1.27) in one direction—(reduction of pyruvate to lactate) according to Sjödin (1976) and phosphorylase (E.C.2.4.1.1) according to Lowry (1957) as modified by Harrison (1971).

STATISTICS

Conventional statistical methods were utilized to identify the *t*-test and analysis of variance. Significant differences between groups in analysis of variance were further examined according to Armitage (1971).

RESULTS

Effects of body weight and food intake (Table 1)

Compared to sham operated controls (Group IV) body weight was significantly lower in the castrated groups without substitution (Group III). The smaller dose of testosterone (Group II) did not seem to prevent this. The large dose of testosterone (Group I) seemed on the other hand to be sufficient to maintain normal body weight compared to the sham operated control (Group IV).

Comparison of food intake revealed that the castrated rat without substitution (Group III) and the smaller dose of testosterone (Group II) ate significantly less than the rat with the larger dose of testosterone (Group I). Moreover, both groups I and III ate somewhat less than sham operated controls, though the differences were not statistically significant (Table 1).

Fat cell size (Table 2)

The castrated rat in group III had smaller fat cell weights in most of the investigated regions compared to the sham-operated rat. The results

ence in cell weight was found in the caudal tarsal adipose tissue depot. The rats treated by small dose of testosterone (Group II) did not reveal any significant differences in fat cell weight compared to sham-operated rats.

4. Fiber composition and muscle enzyme activity (Figs 1 and 2)

Castrated rats with the large dose of testosterone (Group I) had statistically significantly increased proportion of type IIA fibers in their posterior muscles compared to non-substituted rats. The same trend was found in the rats treated with small dose of testosterone. Castration caused a significant decrease in phosphocreatine and lactate dehydrogenase activities in the posterior portion of the gastrocnemius muscle (Fig. 1). This decrease was also found in rats treated with small dose of testosterone (Group II) but was almost eliminated by the large dose.

DISCUSSION

Although substitution caused significantly increased weight gain in the prepubertal male rats in the study in agreement with earlier studies (Sawyer 1941; Scow 1952; Kochakian et al. 1961; Kral & Trefl 1976). The differences in body weight between the rats with and without substitution were not apparent during the first 5 weeks of study. It is interesting to note that these differences occurred at the time when the differences in body weight between

Fig. 2. Mean food intake in castrated sham operated and testosterone treated rats

| Period of treatment | Food consumed g/day/rat (mean \pm S.D.) |
|---------------------------------------|---|
| Group I: Castrated large dose (n=10) | 19 \pm 3 |
| Group II: Castrated small dose (n=10) | 15 \pm 2 |
| Group III: Castrated serum oil (n=10) | 15 \pm 2 |
| Group IV: Sham operated—serum oil | 18 \pm 3 |
| Period of variance | |
| 1-5 days | 0.01 |
| 5-10 days | 0.01 |
| 10-15 days | 0.001 |
| 15-20 days | 0.001 |
| 20-25 days | 0.001 |

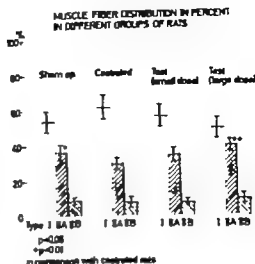


Fig. 1. The distribution (in percent) of slow twitch (type I) and fast twitch (type II A and type II B) muscle fibers in sham operated, castrated and testosterone treated (small and large dose) rats (9 rats in sham operated, otherwise 10 rats in each group). Significance calculated in comparison with castrated rats.

the sexes usually occur (at 7-8 weeks of age). Thus it is tempting to conclude that the differences in body weight between the sexes may be due to androgenic hormones. In view of the well known protein anabolic effects of testosterone even in intact sexually mature animals, the debut of the sex differences in growth and body weight at puberty may rather be interpreted as a result of both a stimulatory effects of testosterone in the male and an inhibitory effect of estrogens in the females. In contrast to male rats gonadectomized female rats gain more weight than intact animals (Kakolewski et al. 1968). Moreover neonatal female rats when functionally oophorectomized by single injections of androgen, gain weight more rapidly than normal females (Swanson et al. 1963).

As judged by generally diminished fat cell weight castration seems to be associated with lower body fat content in comparison with normal or castrated and testosterone treated rat. Differences in the degree of fat cell weight decrease after castration changed the normal regional distribution pattern of adipocytes. This is explained by uneven emptying of fat cells. After castration the epididymal adipocytes decreased by 5% while subcutaneous adipocytes only by 9%. The lack of testosterone effect seems to be responsible for these changes as the

Table 1

| Group | Mean weight ing (\pm S.D.) | Analysis of variance | |
|--|-------------------------------------|----------------------------|--------|
| | | Groups | P |
| I Castrated—large doses of testosterone ($n=10$) | | | |
| Initial weight | 96.9 \pm 5.4 | I-II | <0.001 |
| Final weight | 349.3 \pm 16.8 | I-III | <0.001 |
| | | I-IV | n.s. |
| II Castrated—small doses of testosterone ($n=10$) | | | |
| Initial weight | 96.5 \pm 6.5 | II-III | <0.001 |
| Final weight | 301.1 \pm 16.4 | II-IV | <0.001 |
| III Castrated—sesam oil ($n=10$) | | | |
| Initial weight | 93.9 \pm 4.2 | III-IV | <0.001 |
| Final weight | 304.1 \pm 14.2 | | |
| IV Shamop—sesam oil ($n=9$) | | | |
| Initial weight | 96.4 \pm 4.2 | | |
| Final weight | 350.0 \pm 19.5 | | |

The size of the larger dose calculated from recent data on testosterone secretion rates in rats (de Jong et al. 1973; Free et al. 1973) was chosen to simulate physiological levels of this hormone. All rats were weighed once a week and the food reservoir three times a week to evaluate food intake. After 1 week the animals were fasted over-night, weighed and sacrificed by carotid exsanguination in a well-defined pentobarbital narcosis (50 mg/kg) (Kral 1974). Fat depots from different anatomical regions were removed according to procedures previously described by Krokiewicz & Björntorp (1975) weighed and analysed for determination of fat cell size according to Sjöstrom et al. (1971).

Muscle tissue Immediately after sacrifice before dissection of fat depots two specimens each of the white part of the gastrocnemius and the soleus muscle were dissected. Access to the soleus muscle was gained through a sagittal incision from the most distal part of the Achilles tendon up to the level of the knee. After division of the tendon the gastrocnemius was dissected free from the soleus thus uncovering the intermuscular fat pad which was removed. Subsequently the soleus was prepared by trimming off excess fat and fascia before dividing it into two equal samples.

The white portion of the gastrocnemius was localized at the level of the knee after incision of the km. Excess subcutaneous fat and fascia was trimmed off before excision of muscle tissue. Macroscopically this muscle was homogeneously white in appearance in contrast to the dark red color of the soleus. Microscopy was not performed at this stage.

The samples were frozen directly in liquid nitrogen and stored at -80°C until analyzed for determination of enzyme activity and muscle fiber distribution. One portion of the muscle specimens was cut in a cryostat at -20°C and stained for myofibrillar ATP-ase after preincubation pH 10.3 for classification of type I (slow) and type II (fast) fibres. For classification of the subgroups type IIA (fast

twitch high oxidative FTH) and type IIB (fast twitch low oxidative FTH) both NADPH staining (Novikoff 1961) and ATP-ase staining with preincubation at pH 10.3 was used (Brooke & Kaiser 1970).

After homogenization and weighing in 200 μl of 0.1 M Tris-HCl buffer pH 7.4, a portion of the sample was further diluted to buffer 20 μl aliquots were added to 1 ml of test media. Enzyme activity determinations were performed by fluorometric techniques using a Farrand fluorometer (Farrand Optical Co. New York). The reactions were catalyzed by the enzymes under investigation coupled to NAD/NADP linked reactions, and the changes in NADH or NADPH were monitored according to principles outlined by Lowry (1977).

The measurements were made under V_{max} conditions with an excess of substrate. By running serial curves with known amounts of fluorescence the formal readings could be quantified.

The enzymes investigated in the present study were Mg²⁺ stimulated ATP-ase (E.C.3.6.1.4) (Thompson 1976), lactate dehydrogenase (E.C.1.1.1.27) in oxidation—reduction of pyruvate to lactate according to Lohman (1976) and phosphorylase (E.C.2.4.1.1) according to Lowry (1957) as modified by Harrison (1971).

STATISTICS

Conventional statistical methods were utilized including a *t*-test and analysis of variance. Significant differences between groups in analysis of variance were further examined according to Armitage (1971).

RESULTS

Effects of body weight and food intake (Table 1)

Compared to sham operated controls (Group IV) body weight was significantly lower in the castrated groups without substitution (Group III). The smaller dose of testosterone (Group II) did not seem to prevent this. The large dose of testosterone (Group I) seemed on the other hand to be sufficient to maintain normal body weight compared to the sham operated controls (Group IV).

Comparison of food intake revealed that the castrated rats without substitution (Group III) and the smaller dose of testosterone (Group II) ate significantly less than the rats with the larger dose of testosterone (Group I). Moreover, both groups I and II ate somewhat less than sham operated controls, though the differences were not statistically significant (Table 1).

Fat cell weight (Table 2)

The castrated rat in group III had smaller fat cell weight in most of the investigated regions compared to the sham operated controls. In the

ice in cell weight was found in the caudal meso adipose tissue depot. The rats treated with a small dose of testosterone (Group II) did not show any significant differences in fat cell compared to sham-operated rats.

fiber composition and muscle enzyme (Figs 1 and 2)

castrated rats with the large dose of testosterone (Group I) had statistically significantly lower proportion of type IIA fibers in their caudal muscles compared to non-substituted rats. The same trend was found in the rats treated with the small dose of testosterone.

castration caused a significant decrease in phosphocreatine and lactate dehydrogenase activities in the portion of the gastrocnemius muscle (Fig. 1). A decrease was also found in rats treated with small dose of testosterone (Group II) but was largely eliminated by the large dose.

DISCUSSION

castration without substitution caused significantly reduced weight gain in the prepubertal male rats in line with agreement with earlier studies (Kral & Tisell 1976).

the differences in body weight between the castrated and without substitution were not apparent during the first 5 weeks of study. It is interesting to note that these differences occurred at the time when the differences in body weight between

2. Mean food intake in castrated sham operated and testosterone treated rats

| Groups of treatment | Food consumed g/day/rat (mean \pm S.D.) |
|--|---|
| Group I: Castrated large dose (n=10) | 19 \pm 3 |
| Group II: Castrated small dose (n=10) | 15 \pm 2 |
| Group III: Castrated sham op. (n=10) | 15 \pm 2 |
| Group IV: Sham operated—control (n=10) | 18 \pm 2 |
| Days of variance of intake day/rat | |
| 1-4 | 0.01 |
| 5-8 | 0.01 |
| 9-12 | 0.001 |
| 13-16 | 0.001 |
| 17-20 | 0.001 |

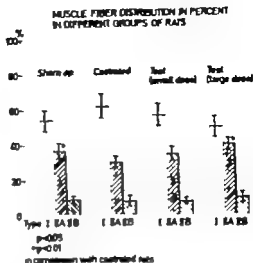


Fig. 1. The distribution (in percent) of slow twitch (type I) and fast twitch (type II A and type II B) muscle fibers in sham operated, castrated and testosterone treated (small and large dose) rats (9 rats in sham operated, otherwise 10 rats in each group). Significance calculated in comparison with castrated rats.

the sexes usually occur (at 7-8 weeks of age). Thus it is tempting to conclude that the differences in body weight between the sexes may be due to androgenic hormones. In view of the well known protein anabolic effects of testosterone even in intact sexually mature animals, the debut of the sex differences in growth and body weight at puberty may rather be interpreted as a result of both a stimulatory effects of testosterone in the male and an inhibitory effect of estrogens in the females. In contrast to male rats gonadectomized female rats gain more weight than intact animals (Kukolewaki et al 1968). Moreover neonatal female rats when functionally oophorectomized by single injections of androgen gain weight more rapidly than normal females (Swanson et al 1963).

As judged by generally diminished fat cell weight, castration seems to be associated with lower body fat content in comparison with normal or castrated and testosterone treated rat. Differences in the degree of fat cell weight decrease after castration changed the normal regional distribution pattern of adipocytes. This is explained by uneven emptying of fat cells. After castration the epididymal adipocytes decreased by 55% while subcutaneous adipocytes only by 9%. The lack of testosterone effect seem to be responsible for these changes as the

Table 3

| Group | Fat cell weight in different regions of adipose tissue mean \pm SE | | | |
|--|--|-----------------|-----------------|-----------------|
| | Perirenal | Inguinal | Epididymal | Caudal |
| I Castrated large dose of testosterone (n=10) | 0.54 \pm 0.05 | 0.30 \pm 0.04 | 0.40 \pm 0.04 | 0.41 \pm 0.05 |
| II Castrated small dose of testosterone (n=10) | 0.50 \pm 0.06 | 0.31 \pm 0.04 | 0.35 \pm 0.04 | 0.37 \pm 0.04 |
| III Castrated injected with sesam oil (n=10) | 0.37 \pm 0.04 | 0.22 \pm 0.04 | 0.27 \pm 0.04 | 0.25 \pm 0.04 |
| IV Sham operated (n=9) | 0.55 \pm 0.06 | 0.25 \pm 0.04 | 0.37 \pm 0.04 | 0.47 \pm 0.05 |
| Analysis of variance | P | P | P | P |
| I-II | n.s. | n.s. | n.s. | n.s. |
| I-III | <0.01 | n.s. | <0.01 | <0.01 |
| I-IV | n.s. | n.s. | n.s. | n.s. |
| II-III | n.s. | n.s. | n.s. | <0.01 |
| II-IV | n.s. | n.s. | n.s. | n.s. |
| III-IV | <0.01 | n.s. | <0.05 | <0.01 |

large dose of testosterone restored the normal distribution pattern of fat cell weight

Similar selective decrease of fat cell size in epididymal depot after castration was recently

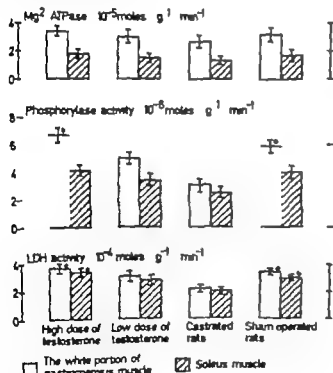


Fig. 2. The activity of the Magnesium stimulated ATPase, Phosphorylase and Lactate Dehydrogenase (LDH) in the white portion of gastrocnemius muscle and (predominantly red) soleus muscle. The activities were estimated in castrated and testosterone treated (high and low dose) rats (9 rats in sham operated, otherwise 10 rats in each group). Significance calculated in comparison with castrated rats.

found by Kral & Tisell (1976). As suggested by the author, the localization of the epididymal fat suggests a role in the reproductive function of the testis and epididymis which could explain a hypersensitivity of the adipocytes in this depot due to lack of testicular steroids.

Castration caused a decrease in the proportion of FTH (type II A) fibres in white gastrocnemius muscle. This change in relative distribution pattern could be reversed by testosterone administration. Testosterone administration to female quinea pigs has been previously found to increase both muscle weight, fiber size and relative proportion of fast twitch white muscle fibers (Gutman & Haddad 1970). In this respect castration without substitution caused "a feminization" of the muscle fiber distribution pattern.

The effect of testosterone on phosphorylase activity is in accordance with previously described increase of total muscle glycogen content after testosterone treatment in rats (Adolf von 1971). According to Newsholme (1978), the maximum rate of anaerobic glycolysis in any given muscle can be predicted from the maximum activity of phosphorylase (or phosphofructokinase).

Thus higher glycolytic activity, higher glycogen content and the postulated higher rate of glycogenolysis in testosterone substituted rats can be seen as in accordance with previously described testosterone effect on strength and muscle mass (Ward 1973). As the reciprocal relationship of phosphorylase and oxidative enzymes in skeletal muscle has been postulated previously (Dubowitz & Pette 1960), the lack of biochemical changes in testosterone

work is in harmony with the concept of the testosterone castration effect on the high (type II) fast twitch muscle fibers.

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Table 3

| Group | Fat cell weight in different regions of adipose tissue mean SE | | | |
|---|--|-----------------|-----------------|-----------------|
| | Perirenal | Inguinal | Epididymal | Caudal |
| I. Castrated large dose of testosterone ($n=10$) | 0.54 ± 0.05 | 0.30 ± 0.04 | 0.40 ± 0.04 | 0.41 ± 0.05 |
| II. Castrated small dose of testosterone ($n=10$) | 0.50 ± 0.06 | 0.31 ± 0.04 | 0.39 ± 0.04 | 0.37 ± 0.04 |
| III. Castrated injected with sesame oil ($n=10$) | 0.37 ± 0.04 | 0.21 ± 0.04 | 0.71 ± 0.0 | 0.25 ± 0.0 |
| IV. Sham operated ($n=9$) | 0.55 ± 0.06 | 0.35 ± 0.04 | 0.37 ± 0.04 | 0.41 ± 0.05 |
| Analysis of variance | P | P | P | P |
| I-II | $n.s.$ | $n.s.$ | $n.s.$ | $n.s.$ |
| I-III | <0.01 | $n.s.$ | <0.01 | <0.01 |
| I-IV | $n.s.$ | $n.s.$ | $n.s.$ | $n.s.$ |
| II-III | $n.s.$ | $n.s.$ | $n.s.$ | <0.01 |
| II-IV | $n.s.$ | $n.s.$ | $n.s.$ | $n.s.$ |
| III-IV | <0.01 | $n.s.$ | <0.05 | <0.01 |

large dose of testosterone restored the normal distribution pattern of fat cell weight.

Similar selective decrease of fat cell size in epididymal depot after castration was recently

found by Kral & Tittel (1961). As reported by the author "the localization of the epididymal fat suggests a role in the reproductive function of the testis and epididymis which could explain the sensitivity of the adipocytes in this depot to the lack of testicular steroids".

Castration caused a decrease in the proportion of FTH (type IIa) fibres in white gastrocnemius muscle. This change in relative distribution pattern could be reversed by testosterone administration. Testosterone administration to female quinea pigs has been previously found to increase both muscle weight, fiber size and relative proportion of both white muscle fibers (Gutman & Huxley, 1970). In this respect castration without subsequent testosterone treatment caused a feminization of the muscle fiber distribution pattern.

The effect of testosterone on phosphorylase activity is in accordance with previously demonstrated increase of total muscle glycogen content after testosterone treatment in rats (Adolf & Auer, 1971). According to Newholme (1974) the maximum rate of anaerobic glycolysis in any given muscle is predicted from the maximum activity of phosphorylase (or phosphofructokinase).

Thus higher glycolytic activity, higher glycogen content and the postulated higher rate of glycolysis in testosterone substituted rats can be seen as in accordance with previously described testosterone effect in strength and muscle fiber type composition. The reciprocal relationship between phosphorylase and oxidative enzyme in skeletal muscle has been postulated previously (Dubowitz & Packer, 1960) the lack of which changes in "red" and "white" muscle.

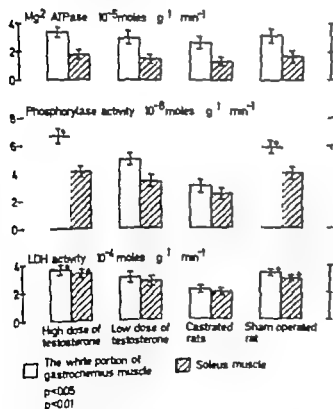


Fig. 1. The activity of the Magnesium-titrated ATPase, Phosphorylase and Lactate Dehydrogenase (LDH) in the white portion of gastrocnemius muscle and (predominantly red) soleus muscle. The activities were estimated in sham-operated, castrated and testosterone-treated (high and low dose) rats (9 rats in sham-operated, otherwise 10 rats in each group). Significance calculated in comparison with castrated rats.

Human renal cytoplasmic carbonic anhydrase

levels and kinetic properties under near physiological conditions

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WISTRAND I. J. Human renal cytoplasmic carbonic anhydrase. Tissue levels and kinetic properties under near physiological conditions. *Acta Physiol Scand* 1980, 109: 39-48. Received 24 Oct. 1979. ISSN 0001-6772. Department of Medical Pharmacology, Biomedical Center, University of Uppsala, Sweden.

The cytoplasmic form of human renal carbonic anhydrase, CA-C (carbonate dehydratase EC 4.2.1.1) purified by affinity chromatography was characterized kinetically at 37°C in 20 mM N-methylimidazole buffer, pH 7.1 using pH-indicator stopped-flow technique. Under these conditions the rate constants for the uncatalyzed hydration of CO₂ and dehydration of H₂CO₃ were 0.12 s⁻¹ and 0.60 s⁻¹ respectively. The kinetic parameters for CA-C were found to be: Hydration reaction, K_m=11.8 mM, V/[E]₀=10.6 · 10³ s⁻¹; dehydration reaction, K_m=70 mM, V/[E]₀=5 · 10³ s⁻¹. In the hydration reaction CA-C was non-competitively inhibited by acetazolamide, K_i=16 μM, sulfisulfamide, K_i=8 μM and chlorothalidate, K_i=1 μM. The levels of immunoreactive CA-C in cortex medulla and papilla of perfused donor kidneys were 1.3, 1.0 and 0.6 mg enzyme protein/g tissue protein respectively which corresponded well with the levels measured catalytically. The erythrocytic form, HCA-B, was also detected immunohistochemically (~0.1 mg/g protein) but is thought to be contaminant. Calculations indicated that the uncatalyzed hydration of CO₂ in the tubular cells can support 17 or 0.7% of the rate of urine acidification dependent on whether the cellular alkaline pH-disequilibrium during acid secretion is 0.1 or 0.01 pH units, respectively. CA-C accelerates the hydration rate 6 800-fold which enables the cell to sustain high rates of proton generation while maintaining near CO₂-equilibrium and maximal buffering capacity. Even at an assumed pH-disequilibrium of only 0.01 pH-unit, CA-C is present in 30-fold excess of apparent physiological needs. When the enzyme is inhibited the rate of the uncatalyzed reaction increases, which partly overcomes the effect of inhibition.

Key words: Kidney carbonic anhydrase, urine acidification, bicarbonate reabsorption, electrophoretic affinity chromatography, radioimmunoassay, stopped-flow technique.

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Key words: Kidney, carbonic anhydrase, uric acidification, bicarbonate reabsorption, diabetes, affinity chromatography, radioimmunoassay, stopped-flow technique.

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plasmic enzyme was isolated in highly purified form and shown to be probably identical with the high activity (with respect to CO_2) isoenzyme HCA-C found in the erythrocytes (Wistrand et al. 1975). This enzyme here called CA-C has been isolated from cortex, medulla and the papilla and appears to constitute the only soluble type of cytoplasmic carbonic anhydrase in the kidney (Währstrand & Wistrand 1990). In the present paper the kinetic properties of this pure enzyme was studied at 37°C under near physiological conditions. The access to a new radioimmunosorbent technique for assaying the levels of this enzyme in human tissues also offered a possibility to determine its levels in the various regions of the kidney. The data obtained were used to estimate the quantitative role of the enzyme for the kidney function using an approach somewhat different from those used by the above cited authors but similar to the one used by Davies (1948) for the acid secreting cells of the stomach.

MATERIALS AND METHODS

Preparation of kidney extracts

The kidneys used were fresh donor kidney ready for transplantation. There was no history of renal disease. Thiazides or other drugs of importance had not been administered to the donors as far as it is known. Immediately after removal the kidneys were perfused with 400–100 ml of cold Ringer-dextran type of solution (Perfadex® Pharmacia, Uppsala, Sweden) containing 10 million IU benzylpenicillin, 50 ml 2% lidocaine (Xylocain® Astra Södertälje, Sweden) and 5000 IU heparin per 1000 ml solution. This was followed by perfusion with 400–1000 ml of 10% invertose solution (Inverdex, Pharmacia, Uppsala, Sweden) to which sodium bicarbonate had been added. The perfusion pressure was 140 mm Hg. The warm ischaemic period varied between 30 and 60 min. The kidneys were stored at 4°C and tissue preparation started within 4 h of perfusion. Slices were taken from the cortical, medullary and papillary region and gently homogenized in ice-cold 0.2 M sucrose. An aliquot of the homogenate was taken for analysis of protein by the method of Lowry et al. (1951) and of carbonic anhydrase isoenzymes (see below). The homogenate was centrifuged at 100 000 $\times g$ for 60 min. The particulate fraction and the fluffy top layer were removed. The supernatant was dialyzed against 10 mM HEPES buffer pH 8.3, 200 μM EDTA and frozen. Its content of carbonic anhydrase isoenzymes and hemoglobin was then determined. Hemoglobin was assayed by the cyan-methemoglobin method (Hampen & Zijlstra 1961). Due to the presence in kidney of other iron porphyrin substances this method probably overestimates the content of hemoglobin and therefore also the amount of contaminating blood in the kidney tissues by about 50% as found by Funakoshi & Deutsch (1971).

Preparation of purified renal soluble carbonic anhydrase

The cytoplasmic soluble enzyme from the pooled supernatants was purified by affinity chromatography as described previously (Wistrand et al. 1975, Währstrand & Wistrand 1980). The erythrocyte isoenzymes HCA-C and HCA-B were similarly prepared. The adsorbent was prepared by coupling the inhibitor sulfanilamide to an open activated agarose via a long hydrophilic spacer group according to the method of Sundberg & Persh (1974). The adsorbent is stable, effective and selective for both (HCA-B) and high (HCA-C) activity forms of carbonic anhydrase isoenzymes. The unspecific adsorption is low and the enzymes eluted are highly purified. The purified enzymes were stored in a refrigerator as a precipitate in saturated ammonium sulphate. This prevents loss of significant enzyme activity during several months. The enzyme concentration was calculated from the absorbance $A_{215}(\text{cm})$ using a value of 18.7 for mole % weight was taken to be 79 800 (Wistrand et al. 1975).

Kinetic methods

Substrate kinetics. The kinetic properties of the pure renal soluble enzyme were studied at 37°C using the pH indicator optical stopped-flow technique. The procedure was outlined by Khalifah (1971) as follows: using Indicator (p-nitrophenol indicator grade reagent, diluted four times in water) and buffer (N-methylimidazole, E. Merck A.G. Darmstadt, GFR, purified by distillation under reduced pressure) with similar pH values, allowing the reaction to occur during pH-changes of less than 0.06 pH units. The measurements were made in a stopped-flow spectrophotometer (Durrum Inverse Corp., Palo Alto, Calif., USA) equipped with a Teflon flow system, 20 mm optical path length observation chamber and specially made tight drive-magnets suitable for work with gaseous substrates. The temperature was maintained by circulating water from a constant temperature bath around the drive syringes and the mixing reaction chambers. Substrate solution of CO_2 freshly prepared by bubbling 100% CO_2 from a gas cylinder into distilled boiled water at the temperature of the

stopped-flow apparatus. It was diluted with CO_2 degassed water in a syringe to give the required substrate concentration. The CO_2 concentrations were calculated from Henry's law by using the solubility coefficients of CO_2 as given by Harned & Dani (1943). Buffer solutions were prepared freshly from crytal buffer stock for use. The CO_2 concentration in these solutions was determined using a CO_2 -electrode (type 5016, Radiometer, Copenhagen, Denmark). The reaction was initiated by mixing equal volumes of the substrate with the solution containing indicator and enzyme. The strength of the assay mixture was kept constant physiologically by adding sodium sulphate. Sulphate chosen because it has not been incriminated as catalyst or inhibitor of the reaction (Magid 1968, Khalifah 1971). Steady-state initial rates were obtained from the linear portions of the photographed reaction. Measurements were repeated usually three times with the same substrate buffer solutions. The initial rate used in calculation of the kinetic parameters was taken as an average of these determinations. It was corrected for

the uncatalyzed reaction. Each set of the Michaelis-Menten is determined with one enzyme-buffer solution between 6 and 12 different substrate solutions. Michaelis-Menten equation is fitted to the experimental data by an iterative method with the aid of least-squares curve-fitting program, executed on a 386/155 digital computer. The kinetic parameters and their standard deviations were then obtained as found necessary to correct for the back-reaction due to significant amounts of CO_2 in the bicarbonates. This correction is made based on the data for the enzymic dehydration rate in the presence of CO_2 (De Voe & Kitzkowski 1961).

Linear Inhibition of the enzyme by sulfinamide (Sigma C, St. Louis, Mo, USA) and thiazide (Merck, Sharp & Dohme, Rahway, N.J., USA) and sodium chloride as titrated inhibitor concentrations and ~ 3 substrate concentrations.

The data resulted in straight lines and indicated competitive type of inhibition (Fig. 2) when plotted according to the graphic method of Dixon (1953). It was felt justified to use the non-linear equation

$$v = \frac{V_{\text{max}}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}}$$

where the concentration of inhibitor which reduced the reaction rate by half I_{50} , and its standard deviation are the measured initial rates in the presence or absence of total inhibitor concentration I respectively. Inhibitor constant, K_i , was obtained from the relation $I_{50} = K_i$, where $[E]_0$ is the total enzyme concentration.

Activity of carbonic anhydrase islets in the renal cortex of the kidney

The kidney proteins were assayed in the renal cortex of the kidney by radio-immunoassay (RIA) (Wistrand et al. 1979) using antibodies selective for human erythrocyte isoenzymes HCA-C (HCA-C) and HCA-B (anti-HCA-B). The antibodies produced as previously described by Wistrand & Wistrand (1979). Antibodies against the purified renal enzyme were also produced and found to react like anti-HCA-C. This would be expected since HCA-C and the renal enzyme are antigenically and otherwise identical (Wistrand et al. 1979). The sensitivity of this method is 0.2 ng of enzyme per mg of tissue fluid and the precision is $\pm 10\%$. Recovery experiments have shown that the accuracy of the method have shown for several human tissues that the method detects all and $\sim 80\%$ of HCA-C and HCA-B respectively (Wistrand & Öberg 1979).

The activity of the renal isoenzymes was assayed by the changing pH-method of Philpott & Philpott (1964). This involves the determination of time to lower pH (10 to 7.4) of 1 ml of carbonate buffer as a change in color of phenol red at 0°C in 7 ml. One enzyme unit is the amount of enzyme that decreases the reaction time by half. The activity was also titrated (Kernohan 1965) by use of the strong

Table 1 Hydration and dehydration kinetic parameters for human erythrocyte carbonic anhydrase purified by affinity chromatography

Conditions of test: 25 mM N-methylimidazole buffer with added sodium sulphate to keep the ionic strength at 0.15 M, pH 7.1 \pm 0.03, 0.01% peptone, 50 μM p-nitrophenol, $37^\circ\text{C} \pm 0.1^\circ$. $[E]_0$ was 0.04 μM . Six different inhibitor concentrations were tested against 1.6 and 1.7 mM CO_2 or 9.0 and 30 mM HCO_3^- . The inhibitor was present in both directions and was added with enzyme in the buffer syringe about 2 min before the reaction started. The parameters are given with their 95% confidence limits within bracket. k_2 for the uncatalyzed hydration reaction was 0.12. The dehydration second-order rate constant was 17 M $^{-1}$ s $^{-1}$. When multiplied by the ionization constant for H_2CO_3^* , $K_{\text{H}_2\text{CO}_3^*}$, taken to be 3.5×10^{-4} M we obtain $k_{\text{H}_2\text{CO}_3^*}$ of 60.

| Kinetic parameter | Hydration | Dehydration |
|--------------------------------------|------------------------|---------------------|
| K_m , mM | 11.8 (6.9–16.7) | 70 |
| $10^{-3} \sqrt{V/[E]_0}$ | 10.6 (8.1–13.1) | 5 |
| K_i , sulfinamide μM | 6.4 (5.0–7.8) | 9.1 (6.5–11.7) |
| K_i , chlorothiazide μM | 0.96 (0.72–1.20) | 0.99 (0.85–1.13) |
| K_i , acetazolamide μM | 0.016 (0.005–0.038) | – |
| K_i , sodium chloride mM | >200 | – |

($K = M$) inhibitor ethoxzolamide (Upjohn Ltd, Kalamazoo, Mich, USA). Each molecule of carbonic anhydrase binds one molecule of ethoxzolamide. These titrations are electrochromic, changing pH-method (Wistrand & Klemm 1977) was used to assay the enzyme activity before and after graded inhibition (details are given in the legend of Fig. 3).

RESULTS

Kinetics of the uncatalyzed and catalyzed hydration and dehydration reactions at 37°C

Uncatalyzed reactions. The rate constants for the hydration of CO_2 and the dehydration of H_2CO_3^* at 37°C differ considerably in the literature as summarized by Garg & Maren (1977). Our values for k_{CO_2} and $k_{\text{H}_2\text{CO}_3^*}$ (Table 1) agree with those previously reported for the stopped-flow technique where buffered media were used. Secondary phosphate has been claimed (cf. Denward & Williams 1966) to catalyze both the hydration and the dehydration reaction. Other investigators (cf. Magid & Turbeck

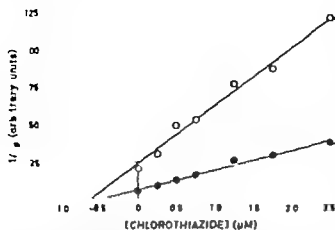


Fig. 1 Inhibition of purified human renal cytoplasmic carbonic anhydrase by chlorothiazide. Stopped-flow method. Hydration reaction. Enzyme $[E]_0 = 0.04 \mu\text{M}$. Inhibitor was present in both drive syringes and mixed with the enzyme 10 min before assay. The assay medium was 25 mM N-methylimidazole buffer pH 7.1, $50 \mu\text{M}$ p-nitrophenol, 0.01% peptone, 37°C . Ordinate reciprocal initial velocity arbitrary units, abscissa concentration of chlorothiazide. $[\text{CO}_2] = 1.56 \text{ mM}$ (unfilled rings) or 7.34 mM (filled rings).

1968) found it to be without effect. In our system the rate constants were not significantly different when measured in 25 mM phosphate or N-methylimidazole buffers at 37°C . It is therefore not likely that phosphate has a physiologically significant effect on the uncatalyzed hydration of CO_2 or dehydration of H_2CO_3 .

Catalyzed reactions. When data of Table 1 are compared with previous kinetic data collected at 25°C (Wistrand et al. 1973) the only difference between the values of 25 and 37°C was the 1.5–2 times larger turnover numbers at 37° . The values for k_m however were not significantly changed by the temperature rise. The data agree qualitatively with those of Kermohan (1977) who has made a systematic study on the effect of temperature on the kinetics of the purified bovine erythrocyte enzyme. The data indicate that the observed increase in the enzymatic reaction rate is due to an increase in the turnover number whereas K_m is rather invariant with temperature.

N-methylimidazole has been reported by Elhalafah (1971) to be non-inhibitory in the hydration reaction of HCA-C. Strong phosphate buffers ($>50 \text{ mM}$) have been reported to inhibit human erythrocyte enzymes (Kermohan 1965) in the hydration reaction although this was ascribed to impurities in the phosphate buffer used. In contrast to these findings

Christenson & Magid (1970) found that phosphate has a net activating effect on HCA-C in the hydration reaction at pH 7.3. In the present experiments a 25 mM phosphate buffer was not inhibitory or activating, since we observed no significant difference between the k_m and turnover number of the enzyme when compared with the values collected in the N-methylimidazole buffers at 25°C and 37°C . It therefore appears unlikely that the phosphate concentrations *in vivo* will affect the catalytic activities of the renal enzyme.

The kinetic parameters of the dehydration reaction (Table 1) agreed reasonably well with the calculated (DeVoe & Kristalowsky 1961) from hydration kinetic parameters and the Haldane relation. The value for k_m agrees with that previously collected at 25°C in phosphate buffers by Gehr and Edsall (1964) and by Wistrand et al. (1973). The results were similar to those seen in the hydration reaction, i.e. raising the temperature increased the turnover number without affecting the k_m .

Inhibitor kinetics. Sulfanilamide and chlorazide were tested in the hydration and the dehydration reactions giving curves which indicated a competitive type of inhibition (Fig. 1). The inhibition by acetazolamide was non-competitive in the hydration reaction. Raising the temperature from 25 to 37°C increased the k_1 for sulfanilamide 2–3 fold. The effect of temperature on the inhibition by sulfanilamides has not been studied systematically but seems to vary for different sulfonamides and perhaps also with the type of enzyme (Kern Willey 1968). Chloride was not inhibitory ($K_i > 1 \text{ mM}$).

Levels of carbonic anhydrase in different regions of the kidney

The immunoassayable levels of CA-C and CA-B in the supernatants of kidney no. 15, 16 and 17 were similar to those of the other kidneys where the enzymes were instead titrated on the whole homogenate (see Table 2). In kidney no. 6 and 8 titrations were done on the supernatant as well as on the homogenate. The average ratio of the values for CA-C of the supernatant and homogenate was 1.1 ± 0.1 (S.D. $n=4$). These data therefore indicate that the enzymes in the homogenate remained in solution after centrifugation and were not adsorbed to the particulate fraction.

The values for CA-C in cortex and medulla expressed as mg enzyme in per g tissue protein

Immunisable levels of carbonic anhydrases B and C in various regions of the human kidney
 (Hb as determined by the cyan-methemoglobin method of Kampen & Zijlstra (1961))

Level in mg per g of extractable protein of homogenate

| Cortex | | | Medulla | | | Papilla | | |
|--------|------|-----|---------|------|-----|---------|------|------|
| CA-B | CA-C | Hb | CA-B | CA-C | Hb | CA-B | CA-C | Hb |
| 0.014 | 1.40 | | | | | | | |
| 0.027 | 0.96 | | 0.030 | 0.58 | | 0.078 | 0.76 | |
| | | | 0.330 | 1.78 | | 0.100 | 0.21 | |
| 0.022 | 1.60 | | | | | | | |
| 0.200 | 1.50 | | 0.018 | 0.68 | | 0.095 | 0.50 | |
| 0.140 | 1.36 | | | | | | | |
| 0.260 | 1.57 | | | | | | | |
| 0.124 | 0.96 | 5.6 | 0.098 | 0.86 | 7.2 | 0.094 | 1.03 | 8.0 |
| 0.078 | 1.45 | 7.4 | 0.044 | 1.29 | 5.2 | 0.056 | 0.94 | 7.4 |
| 0.017 | 0.99 | 4.3 | 0.027 | 0.66 | 5.1 | 0.150 | 0.42 | 26.0 |
| 0.098 | 1.31 | | 0.091 | 1.00 | | 0.096 | 0.64 | |

Average catalytic activities in cortex, medulla and papilla of these donor kidneys were 242, 194 and 157 enzyme units respectively as determined by the indicator changing pH-method of Philpott & Philpott (1936)

gibly lower than those 1.71 and 1.73 mg/g of by Fuzakowski & Deutsch (1971). This is explained by less contribution of HCA-C to the total activity of red cells in our perfused kidney. The values for CA-C were highest in cortex and medulla and papilla in agreement with the order of catalytic activities found in these regions (Table 2). The same order has been found for renal tubular cells, dissected out from the different parts of the single human nephron (Malmgren et al 1968).

Our values correspond to 0.1, 0.1 and 0.06 mg/g of wet tissue of cortex, medulla and papilla respectively. Since the specific activity of pure CA-C is 1520 enzyme units per mg (Wistrand 1975) it may be calculated that cortex, medulla and papilla should contain 182, 162 and 91 enzyme units per g wet tissue respectively. These values are in well with those determined experimentally on these regions (Table 2), using the same catalytic method for measuring the enzyme units. This indicates that the majority of antigenically active CA-C is catalytically active. This should rule out the possibility that the kidney contains other high-activity forms of the enzyme immunochemically different from CA-C. Moreover, the titration of the active catalytically active enzyme sites by use of a strong inhibitor ethoxzolamide gave values in good agreement with those of the immunochemical method. The average ratio between values of ethoxzolamide and antibody titrations

of 4 supernatants was 1.00 ± 0.3 (S.D.). The results of an experiment on the inhibition of the enzyme activity of cortex and medulla of one kidney is seen in Fig. 2. The activity of a fixed concentration of

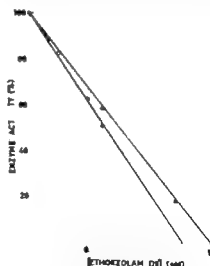


Fig. 2. Titration of renal carbonic anhydrase activity by the inhibitor ethoxzolamide. Electrostatic method. Conditions of test: 10 mM phosphate buffer with 0.1 mM Na_2SO_4 , 0.001 M EDTA, 12.5 mg/ml bovine plasma albumin, $[\text{CO}_2]$ was 3.8 mM, initial pH 7.0, $[\text{E}]_0$ was 1.2 and 0.8 a.u. respectively as determined immunochemically on the supernatants of the cortical (open rings) and the medullary (filled rings) homogenate. Ordinate: activity as percentage of activity in absence of inhibitor. Abscissa: concentration of ethoxzolamide in mM.

enzyme is plotted against the amount of inhibitor added. The intercept on the inhibitor axis gives the value for the moles of enzyme present 1.3 nM in cortex and 1.5 nM in medulla. In this experiment the values were slightly higher than those 0.8 and 1.2 nM respectively obtained by the immunosay.

We believe that all CA-B titrated immunochemically (Table 2) originates from contaminating blood. From the hemoglobin values in the cortex, medulla and papilla of kidneys no. 15, 16 and 17 (Table 2) it may be calculated that the kidneys contain less than 0.8% blood except for the papilla of kidney no. 17 which contained 2.5%. Since whole blood contains 12.1 µg HCA-B and 1.5 µg HCA-C per mg hemoglobin (Wahlstrand et al. 1979) it may further be calculated that all CA-B and less than 10% of CA-C originate from blood. The levels of CA-C were rather similar in all kidneys and between 5–100 times higher than those of CA-B in all regions. The levels of CA-B on the other hand varied strongly but were on an average the same in the different regions. Funakoshi & Deutsch (1971) found no CA-B in cortex but 0.99 mg CA-B/g protein in the medulla of a non perfused autopsy kidney. However these authors reported higher hemoglobin values than found here which makes the correction for blood contamination more difficult. In a recent study (Wahlstrand & Wistrand 1980) only CA-C could be isolated from the medullary and papillary regions of well perfused human donor kidneys.

The presence of enzyme proteins in cortex, medulla and papilla agrees with the finding that carbonic anhydrase activity is found histochemically along the whole human kidney nephron with exception for glomeruli and the straight part of the proximal tubule (Lönnerholm 1975).

DISCUSSION

The kinetic data on enzyme activity in highly diluted hemolysates have been shown to be applicable to conditions inside whole erythrocytes (cf. Itada & Forster 1977). The enzyme activity of intact red cells is moreover inhibited by sulfonamides as can be predicted from inhibition kinetics of the purified isoenzymes in dilute solutions (Wistrand & Bååthe 1968). Thus there is evidence that both substrate and inhibitor kinetics are not changed when the enzyme is enclosed inside a cell membrane.

Direct measurements of carbonic anhydrase activity and its inhibition in intact tubular cells have been reported. Estimates of the potential of carbonic anhydrase to catalyze the CO_2 -reaction in these cells are more uncertain than for the erythrocytes due to the presence of carbonic anhydrase not only in the cytoplasm but also in the renal cell membranes. Little is known about kinetic properties of this membrane-bound enzyme (cf. Wistrand & Kinne 1977). However, recent results from micropuncture studies have indicated that only the cytoplasmic enzyme is involved in the process of proximal tubular renal acidification (Karlsson et al. 1979). This has encouraged us to try to estimate the quantitative role of the cytoplasmic enzyme in renal acidification, using the data on its kinetic properties and cellular concentrations as collected in the present paper.

Calculation of the capacity of the uncatalyzed catalyzed CO_2 reaction to generate protons and neutralize alkali within the tubular cells

We assume that renal tubular cells act as proton sinks (Pitts 1958) leaving an alkaline cell interior to be buffered by the hydration of an unlimited supply of CO_2 . Direct support of this hypothesis is the finding that acid secreting separated cells (Struyvenberg et al. 1968) and rabbit (Pauland 1979) renal tubules are relatively alkaline (titrimetric method) and become even more so about 0.1 unit after inhibition of carbonic anhydrase.

The capacity of the uncatalyzed hydration of CO_2 within the tubular cells to generate protons was calculated from the equation

$$\frac{d[\text{CO}_2]}{dt} = -k_{\text{CO}_2} [\text{CO}_2] + k_{\text{H}_2\text{CO}_3} \frac{[\text{H}][\text{HCO}_3^-]}{K_{\text{H}_2\text{CO}_3}}$$

where k_{CO_2} and $k_{\text{H}_2\text{CO}_3}$ are the rate constants (Table 1) and $K_{\text{H}_2\text{CO}_3}$ is the true ionization constant for carbonic acid taken to be $3.5 \cdot 10^{-7}$ M and *in vivo* conditions (cf. Roughton 1964). Since the present kinetic data were collected at ionic strength and buffer capacity in the physiological range, corrections (Jonsson et al. 1976) for the small difference in buffer capacity between the tubular fluid were thought to be unnecessary. The possible catalytic activity (base catalysis) of the cellular concentration of HPO_4^{2-} has been neglected as discussed in the Results section. $[\text{CO}_2]$ was taken to be 0 mM ($\text{PCO}_2 = 65$ mmHg) and in diff-

in the blood (Do Bosc et al. 1978) is assumed to be 25 mM as measured by Khuri (1979) in the rat late proximal tubule using an ion-exchange bicarbonate microelectrode.

Substituting these values into the equation at which $d\text{CO}_3/\text{dt}=0$ and solving for $[\text{H}^+]$, an equilibrium value for the intracellular pH is

only available data on intracellular pH during secretion are those just cited from the derived method. This pH must be on the alkaline side of equilibrium value since only then is CO_2 absorbed and hydrated inside the cell. From some of the equation it becomes obvious that $d\text{CO}_3/\text{dt}$, of CO_2 absorption—or proton secretion—will depend on the assumed magnitude of alkaline pH disequilibrium within the cell. We take the pH of the secreting cell to be 7.35 (an alkaline pH disequilibrium (not to be confused with the pH-disequilibrium of the luminal fluid (Reicer 1973) of 0.10 pH unit) application of equation shows that

$$L = 0.1 \text{ sec} \quad 0 \text{ mM} + 60 \text{ sec} \\ \frac{25 \text{ mM}}{10} = 50 \mu\text{M} \times \text{sec} \quad \text{or } 3 \text{ mM} \times \text{min}$$

value becomes 0.13 mM \times min. If we instead assume that the cell pH is 7.26, i.e. 0.01 pH unit from the equilibrium pH,

we assume further that the uptake of CO_2 takes place in 200 ml of tissue fluid (2/3 of the weight of kidney). This is a generous estimate but takes account the possibility (Mello-Aires & Mainwaring) that the volume of proton generating tissue is larger than that of tubular cells. Under the assumption of good mixing and diffusivity of the CO_2 it may then be calculated that the un-catalyzed reaction could generate 600 or 1.5 μ moles protons per minute dependent on whether we take an alkaline pH disequilibrium of 0.1 (pH 7.35) or 0.01 (pH 7.26) pH-units respectively.

The rate of the enzyme catalyzed reaction may be calculated from the Michaelis-Menten equation, using the kinetic parameters of the enzyme (Table 1) and concentration (Table 2) in the tubular cell (0.7% of cell volume). The enzyme catalyzed rate was calculated to be 6800 times higher than that of the uncatalyzed rate.

The physiological rate of renal acidification

Under normal condition In a healthy person of 70 kg the kidneys would have to generate 356 mol/min of protons to be able to reabsorb all filtered bicarbonate and excrete the relatively small amounts of titratable acid (Bernstein 1958). Dependent on the alkalinity of the cell this is more than 6 (pH 7.35) or 120 (pH 7.26) times the calculated capacity of the uncatalyzed hydration of CO_2 to produce the number of protons necessary for the neutralization of the intracellular hydroxyl ions. However the need to accelerate the rate of the uncatalyzed hydration of CO_2 is easily met by the potential of carbonic anhydrase to catalyze this rate 6800-fold. This gives a large excess of enzyme in relation to physiological needs. However as discussed above the magnitude of this excess is dependent on the alkaline pH-disequilibrium within the cell. Direct measurements of the CO_2 -equilibrium within the cell during acid secretion are therefore needed, before the quantitative role of the enzyme can be defined more strictly. However the calculations do indicate that the activity of the cytoplasmic enzyme enables the tubular cell to sustain high rates of proton secretion sufficient for all demands of urinary acidifications while maintaining near CO_2 -equilibrium and maximal buffering capacity within the cell. This would appear to be the physiological role of the enzyme.

Under carbonic anhydrase inhibition. Another approach to assessing the quantitative role of the enzyme is to see how graded enzyme inhibition relates to the physiological effects. Bernstein (1958) found that a dose of 10 mg/kg of acetazolamide given orally gives maximal 18% reduction of the bicarbonate reabsorption and excretion of $\text{H}^+ + \text{NH}_4^+$ in humans (see model of Fig. 3). At the peak of this renal effect the concentration of unbound inhibitor in plasma, I_{over} is about 10 μM (Lehmann et al. 1969). In the renal cortex it is probably 3 times higher due to accumulation during active renal secretion of acetazolamide (Maren 1968). If we therefore take I_{over} in the tubular cell to be 20 μM the fractional enzyme inhibition will be 0.9993 as calculated from the relation

$$i = \frac{I_{\text{over}}}{I_{\text{over}} + K}$$

This degree of inhibition would reduce the potential of the enzyme to accelerate the hydration of

enzyme is plotted against the amount of inhibitor added. The intercept on the inhibitor axis gives the value for the moles of enzyme present: 1.3 nM in cortex and 1.6 nM in medulla. In this experiment the values were slightly higher than those 0.8 and 1.2 nM respectively obtained by the immunoassay.

We believe that all CA-B titrated immunochemically (Table 2) originates from contaminating blood. From the hemoglobin values in the cortex, medulla and papilla of kidneys no. 15, 16 and 17 (Table 2) it may be calculated that the kidneys contain less than 0.8% blood except for the papilla of kidney no. 17 which contained 2.5%. Since whole blood contains 17.1 µg HCA-B and 1.5 µg HCA-C per mg hemoglobin (Wåhlstrand et al. 1979) it may further be calculated that all CA-B and less than 10% of CA-C originate from blood. The levels of CA-C were rather similar in all kidneys and between 5–100 times higher than those of CA-B in all regions. The levels of CA-B on the other hand varied strongly but were on an average the same in the different regions. Funakoshi & Deutsch (1971) found no CA-B in cortex but 0.99 mg CA-B/g protein in the medulla of a non perfused autopsy kidney. However these authors reported higher hemoglobin values than found here which makes the correction for blood contamination more difficult. In a recent study (Wåhlstrand & Wistrand 1980) only CA-C could be isolated from the medullary and papillary regions of well perfused human donor kidneys.

The presence of enzyme proteins in cortex, medulla and papilla agrees with the finding that carbonic anhydrase activity is found histochemically along the whole human kidney nephron with exception for glomeruli and the straight part of the proximal tubule (Lönnerholm 1975).

DISCUSSION

The kinetic data on enzyme activity in highly diluted hemolysates have been shown to be applicable to conditions inside whole erythrocytes (cf. Itada & Forster 1977). The enzyme activity of intact red cells is moreover inhibited by sulfonamides as can be predicted from inhibition kinetics of the purified isoenzymes in dilute solutions (Wistrand & Bååthe 1968). Thus there is evidence that both substrate and inhibitor kinetics are not changed when the enzyme is enclosed inside a cell membrane.

Direct measurements of carbonic anhydrase activity and its inhibition in intact tubular cells have been reported. Estimates of the potential of carbonic anhydrase to catalyze the CO_2 -reactions in these cells are more uncertain than for the erythrocytes due to the presence of carbonic anhydrase not only in the cytoplasm but also in the renal cell membranes. Little is known about kinetic properties of this membrane-bound enzyme (cf. Wistrand & Kinne 1977). However recent results from micropuncture studies have indicated that renal cytoplasmic enzyme is involved in the proximal tubular renal acidification (Karlsson et al. 1979). This has encouraged us to try to estimate the quantitative role of the cytoplasmic enzyme in renal acidification using the data on its kinetic properties and cellular concentrations as collected in the present paper.

Calculation of the capacity of the uncatalyzed catalyzed CO_2 -reaction to generate protons and neutralize alkali within the tubular cells

We assume that renal tubular cells actively secrete protons (Pitts 1958) leaving an alkaline cell interior to be buffered by the hydration of an available supply of CO_2 . Direct support of this hypothesis is the finding that acid secreting separated cells (Struyvenberg et al. 1968) and rabbit (Pallard et al. 1979) renal tubules are relatively alkaline (pH method) and become even more so about 0.1 pH unit after inhibition of carbonic anhydrase.

The capacity of the uncatalyzed hydration of CO_2 within the tubular cells to generate protons was calculated from the equation

$$\frac{d[\text{CO}_2]}{dt} = -k_{\text{CO}_2}[\text{CO}_2] + k_{11\text{CO}} \frac{[\text{H}][\text{HCO}_3^-]}{K_{11\text{CO}}}$$

where k_{CO_2} and $k_{11\text{CO}}$ are the rate constants (Table 1) and $K_{11\text{CO}}$ is the true ionization constant for carbonic acid taken to be $3.5 \cdot 10^{-4}$ M under *in vivo* conditions (cf. Roughton 1964). Since the present kinetic data were collected at ionic strength and buffer capacity in the physiological range no corrections (Jonsson et al. 1976) for the small difference in buffer capacity between the buffer in tubular fluid were thought to be necessary. The possible catalytic activity (base catalysis) of the cellular concentration of HPO_4 has been neglected as discussed in the Results section. $[\text{CO}_2]$ was taken to be 0 mM ($\text{PCO}_2 = 65$ mm Hg) and in different

— with the blood (Du Bose et al 1978)
 — assumed to be 25 mM as measured
 — by Khris (1979) in the rat late proximal
 — tubule using an ion-exchange bicarbo-
 — native microelectrode

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 — when $dCO_2/dt=0$ and solving for $[H^+]$
 — equilibrium value for the intracellular pH

— only available data on intracellular pH during
 — secretion are those just cited from the derived
 — method. This pH must be on the alkaline side
 — equilibrium value since only then is CO_2
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 — take the pH of the secreting cell to be 7.35
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 — with the pH-disequilibrium of the luminal
 — Sector 1973) of 0.10 pH unit, application of
 — shows that

$$-0.12 \text{ sec} \quad 2.0 \text{ mM} + 60 \text{ sec}$$

$$\frac{5 \text{ mM}}{10^{-4}} = 50 \mu\text{M} \times \text{sec} \quad \text{or} \quad 3 \text{ mM} \times \text{min}$$

— value becomes $0.13 \text{ mM} \times \text{min}^{-1}$ if we instead
 — take the cell pH is 7.26 i.e. 0.01 pH unit
 — the equilibrium pH

— assume further that the uptake of CO_2 takes
 — in 100 ml of tissue fluid ($\sqrt{3}$ of the weight of
 — kidney). This is generous estimate but takes
 — account the possibility (Mello-Aires & Malnic
 — 1971) that the volume of proton generating tissue
 — is larger than that of tubular cells. Under
 — of good mixing and diffusivity of the
 — ions it may then be calculated that the un-
 — catalyzed reaction could generate 600 or 25 μmoles
 — per minute dependent on whether we
 — an alkaline pH disequilibrium of 0.1 (pH 7.35)
 — 0.01 (pH 7.26) pH-unit respectively

— the rate of the enzyme catalyzed reaction may be
 — calculated from the Michaelis-Menten equation, —
 — the kinetic parameters of the enzyme (Table 1)
 — concentration (Table 1) in the tubular cell
 — or 10.7% of cell volume. The enzyme catalyzed
 — as calculated to be 6800 times higher than
 — of the uncatalyzed rate

The physiological rate of CO_2 acidification

Under normal conditions In a healthy person of 70 kg the kidneys would have to generate 3% mol/min of protons to be able to reabsorb all filtered bicarbonate and excrete the relatively small amounts of titratable acid (Bernstein 1958). Dependent on the alkalinity of the cell this is more than 6 (pH 7.35) or 170 (pH 7.26) times the calculated capacity of the uncatalyzed hydration of CO_2 to produce the number of protons necessary for the neutralization of the intracellular hydroxyl ions. However the need to accelerate the rate of the uncatalyzed hydration of CO_2 is easily met by the potential of carbonic anhydrase to catalyze this rate 6800-fold. This gives a large excess of enzyme in relation to physiological needs. However as discussed above the magnitude of this excess is dependent on the alkaline pH-disequilibrium within the cell. Direct measurements of the CO_2 -equilibrium within the cell during acid secretion are therefore needed before the quantitative role of the enzyme can be defined more strictly. However the calculations do indicate that the activity of the cytoplasmic enzyme enables the tubular cell to sustain high rates of proton secretion sufficient for all demand of urinary acidifications while maintaining near CO_2 -equilibrium and maximal buffering capacity within the cell. This would appear to be the physiological role of the enzyme.

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$$i = \frac{I_{free}}{I_{free} + K_i}$$

This degree of inhibition would reduce the potential of the enzyme to accelerate the hydration of

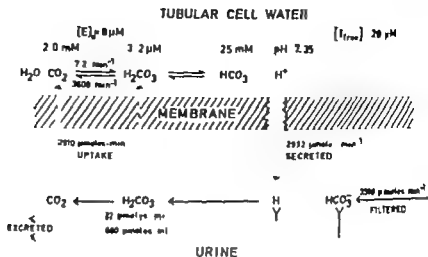


Fig. 3 Model of proton generation and bicarbonate reabsorption in the human renal tubule after an apparent maximally effective dose of acetazolamide. Values on rates of excreted bicarbonate and titratable acid are at ~ 3 h after 10 mg/kg of acetazolamide given orally to healthy humans (Bernsten 1948). Values on $[\text{CO}_2]$ (Du Noe et al. 1978) and $[\text{HCO}_3^-]$ (Khuri 1979) are from direct measurement on rat kidneys. pH was assumed to be 7.35 i.e. 0.1 pH unit on the alkaline side of the equilibrium value of 7.43 (see text). $[\text{HCO}_3^-]$ is the calculated value at pH 7.35. Inhibition of carbonic anhydrase raises the cellular pH by about 0.1 pH unit (Struyvenberg et al. 1968) and thereby increases the rate of the uncatalyzed hydration of CO_2 to 600 $\mu\text{mol min}^{-1}$ (see text). This rate can only support 17% of the absorption of all filtered bicarbonate. However carbonic anhydrase can accelerate the rate 6800-fold. Therefore even if 99.93% of the enzyme is inhibited at an i_{app} of 70 μM (see text) there is still a 5-fold magnification (6800×0.0007) of the uncatalyzed rate which would be enough for the reabsorption of the bicarbonate 910 $\mu\text{mol min}^{-1}$ not excreted in the urine after this dose of acetazolamide. The protons generated intracellularly may be directly secreted (Pitts & Alexander 1945) or used to buffer the alkaline cell interior created by the cell acid secretion as discussed in the text.

CO_2 from 6800-fold to 5-fold (6800×0.0007). However during carbonic anhydrase inhibition the cellular alkaline pH-disequilibrium increases by about 0.1 pH unit (Struyvenberg et al. 1968; Paillard et al. 1979). The uncatalyzed hydration of CO_2 will then generate at least 600 $\mu\text{mol min}^{-1}$ of protons. A 5-fold magnification of this rate would thus still be sufficient to support the absorption of 910 $\mu\text{mol min}^{-1}$ of bicarbonate which continues to take place even after this apparently maximal dose of acetazolamide (Fig. 3). It would therefore appear as if the increased cellular alkalinity makes the cell escape the effects of enzyme inhibition by increasing the rate of the uncatalyzed reaction by about 70-fold. However when 100 μM of acetazolamide was added to the luminal perfusate in micropuncture studies of rat tubules in vivo (Lucci et al. 1979) or in separated rabbit tubules (McKinney & Burg 1977) the proximal bicarbonate reabsorption became reduced by 85–100%. If acetazolamide diffuses freely into the tubular

cell from the luminal side this high concentration would inhibit the enzyme by 99.96% as the rat renal enzyme is kinetically similar to the human enzyme (Wistrand & Wåhlstrand 1977). In this case no enzyme activity should be left in the tubular cell (acceleration less than 5-fold) and only small amounts (<15%) of bicarbonate reabsorbed under these conditions probably reflect the activity of the uncatalyzed hydration of CO_2 . These data support the notion that the reabsorption of all filtered bicarbonate and the excretion of titratable acid is dependent on the hydration of CO_2 and its catalysis by cytoplasmic carbonic anhydrase. Maren (1968) however found that despite they were given increasing doses of diuretics with highly different structures did not excrete more than 70–35% of the filtered bicarbonate. This was taken as evidence that most (65–80%) of filtered bicarbonate is reabsorbed by a very mechanism. On the other hand Mathisen et al. (1979) found a linear relationship between

absorption and log intravenous doses of 1 mg/kg body wt of acetazolamide in dogs section whether all bicarbonate reabsorption is dependent on carbonic anhydrase activity. Therefore still open and will probably not be resolved until direct measurements of enzyme activity in the tubular cells have been done after addition of inhibitors. The use of irreversible reagents for this purpose should be advantageous.

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Dilatation of pial arterial vessels in hypercapnia and in acute hypertension

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AUER, L. M. & JOHANSSON, B. B. Dilatation of pial arterial vessels in hypercapnia and in acute hypertension. *Acta Physiol Scand* 1980, 109: 249-251. Received 30 Oct. 1979. ISSN 0001-6772. Department of Neurosurgery, University of Graz, Austria and Department of Neurology, Sahlgren Hospital, Göteborg, Sweden.

Pial arterial vessels were inspected in hypercapnia (PaCO_2 97 ± 9 (S.E.) mmHg) and in acute hypertension induced by $5 \mu\text{g kg}^{-1}$ noradrenaline i.v. (mean arterial pressure 210 ± 5 mmHg) by means of craniol window. The diameter of arterial vessels with resting diameter of 10 to 150 μm was measured either from photographs or by aid of an image splitting eyepiece. Arterioles with resting diameter $<30 \mu\text{m}$ exhibited the highest degree of dilatation both in hypercapnia and acute hypertension. The mean dilatation was higher in hypertension than in hypercapnia in vessels of all sizes, the difference being statistically significant in arterioles up to 50 μm resting diameter.

Key words: Acute hypertension, hypercapnia, pial artery diameter, craniol window.

Carbon dioxide dilates pial arteries in an old tradition (Donders 1849). A number of studies shows that cerebral blood flow (CBF) increases several hundred percent during hypercapnia in animals and man (for references see Auer 1977). An abrupt increase in blood pressure of a magnitude that surpasses the capacity of the cerebral vessels to autoregulate also increases CBF but, as a concomitant diffuse vasoconstriction is induced up to such high values as in hypercapnia (Auer & Nilsson 1979). This is in agreement with the observation that CBF is heterogeneous in hypertension (Johansson 1974; Bill & Linder 1977). Intravital inspection of pial arteries during hypertension or drug-induced acute hypertension revealed a condition of alternating vasodilation and relative vasoconstriction, so-called 'spasmodic arteries' (Byrom 1954; Rodda & Denrow 1966; MacKenzie et al. 1976; Auer 1977). Dilated parts increase in number when the pressure is kept high for a longer period of time, suggesting that more and more smooth muscle cells in the high intraluminal pressure with time dilatation of cerebral vessels in acute hypertension, which usually leads to a transitory dysfunction of the blood-brain barrier, has been called 'vaso-

'distension' - overstretching or forced dilatation - thus assuming a dilatation more pronounced than that produced by complete relaxation of the smooth muscle cells (Häggendal & Johansson 1972; Johansson, Strandgaard & Lassen 1974). However, the degree of pial vasodilatation present during acute hypertension and hypercapnia respectively has not been compared.

In the present investigation the responses of pial arterial vessels to hypercapnia and to acute hypertension were compared in order to answer the following questions: (a) Is it vessels of the same size that react most to both stimuli? (b) Which dilatation is strongest?

MATERIAL AND METHODS

Ten cats with body weight of 1.5-3.0 kg were anaesthetized with 30 mg kg⁻¹ sodium pentobarbital, intubated endotracheally and ventilated with 3 l mixture of N_2O O_2 with Loeco respirator. The left femoral artery and vein were cannulated for continuous monitoring of blood pressure (Statham transducer P23dB; Hellige Electromonitor, Riksdenski perewriter) and administration of drugs. A craniol window was made in the right parietal region and pial arterial vessels were investigated by measuring diameters from photographs or by aid of an image splitting eyepiece. (For details, see Auer 1977a.)

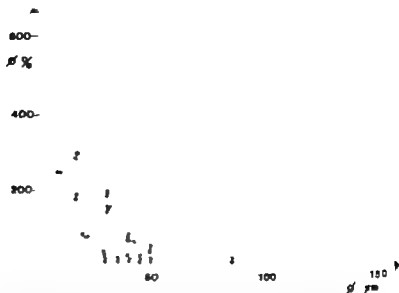


Fig. 1 The percentual vasodilatation in acute hypertension (O) and in hypercapnia (●) plotted against the resting diameter in pial arterial vessels up to 150 μm . The strongest reaction is seen in small vessels both in hypertension and hypercapnia.

Hypercapnia was induced in 5 cats by adding 10–15% CO_2 to the respired gas mixture thus increasing PaCO_2 from 30–41 mmHg (mean 37.8 mmHg) to 73–114 mmHg (mean 96.6 mmHg). The maximum dilatation was measured on arteries and arterioles with a resting diameter of 10 to 150 μm .

Acute arterial hypertension was induced in 5 other cats by intravenous injection of 5 $\mu\text{g/kg}$ norepinephrine. The mean arterial pressure (MAP) rose within 10–20 s from a mean of 98 mmHg to 210 ± 5 mmHg (1.2% increase) returning to normal within about 10 min.

The extent of arteriolar dilatation during hypercapnia and hypertension respectively was compared in 3 groups of arterial vessels with a resting diameter of 1–30 μm , 31–50 μm and 51–150 μm . Differences in the degree of dilatation were evaluated with the Student's *t* test.

RESULTS

During acute hypertension arteries and arterioles dilated mostly sausage string-like and less frequently in a diffuse manner whereas during hypercapnia dilatation was regularly diffuse. Both in hypercapnia and hypertension a reciprocal relation between resting vessel size and degree of dilatation was observed similar to an exponential curve (see also Auer 1978a, b). Thus the smallest vessels exhibited the highest and the largest the lowest degree of dilatation. The percentual vasodilatation plotted against the resting diameter is shown in Fig. 1. It is evident that during acute hypertension (circles) the vessels—especially the dilated portions of “sausage string” vessels—had a larger

diameter than during hypercapnia (squares). In some instances an increase in number or length of the dilated parts was seen as a function of duration of hypertension. The mean diameter was larger in hypertension than in hypercapnia in vessels of all sizes with statistically significant difference in arterioles up to 50 μm resting diameter (see Table 1).

DISCUSSION

Our results thus show that it is the smallest arterial vessels that have the largest dilatation both in hypertension and hypercapnia. The observation that dilatation during hypertension exceeds that in hypercapnia is in agreement

Table 1 The percentual dilatation of arteries of various resting diameters after acute hypertension and hypercapnia

| | Diameter (μm) | | |
|-------------------------|------------------------------|----------------------------|------------------|
| | 0–30 | 31–50 | |
| Hypercapnia | 137 ± 79 ($n=70$) | 45 ± 6 ($n=15$) | |
| Hypertension | $139 \pm 19^*$ ($n=46$) | 111 ± 16 ($n=22$) | |
| Significantly different | | | from hypercapnia |

cept of overstretching and forced dilatation of pial vessels in acute hypertension. The pial arterial vessels are those that react strongly with both dilatation and constriction abruptly increase in blood pressure (Auer). Interestingly it is the same size of meningeal cerebral arterial vessels that shows the most rapid increase in the ratio between media and radius in chronic hypertension in the rat (Sjöberg & Johansson 1979). The strong reaction of pial arterioles with a diameter less than 100 μ m suggests that these vessels are of importance in cerebrovascular resistance—something that is the subject of considerable debate (for review see Purves 1978).

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Patchy intermittent ischemia in renal cortex during tourniquet shock in dog

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The effect of bilateral hindlimb occlusion on the distribution of renal cortical blood flow was investigated in anesthetized dogs. Local blood flow in outer cortex (OCF) and in inner cortex (ICF) was measured by 6 platinum electrodes recording hydrogen gas clearance. Renal blood flow fell insignificantly while hematocrit (Hct) rose from 37 to 47% during the 4 h tourniquet period. After release of the tourniquets average arterial blood pressure fell to about 75% of control, whereas OCF and ICF were proportionately reduced towards 30-40% of control. Hct increased to 58%. Patchy intermittent ischemia in outer and inner cortex was irregularly observed after release of the tourniquets when Hct had returned markedly as previously found during dehydration and endotoxin shock. The sudden shifts in washout rates from single electrodes were not accompanied by corresponding variations in total renal blood flow. Heparinization or α -receptor blocking did not suppress the appearance of abrupt flow changes in this period. However, sudden shifts in local flow were also seen shortly after application of the tourniquets, before Hct had increased, in this phase possibly induced by an increased renal nervous tone. In conclusion: OCF and ICF fell to the same degree during tourniquet shock. Patchy intermittent ischemia was observed in the renal cortex, most frequently at high Hct, but also before Hct had risen.

Key words: Renal blood flow distribution, hydrogen clearance, tourniquet shock, blood flow heterogeneity.

One of the frequent kidney impairment following shock (Rosenberg et al. 1971), little doubt is available on the renal blood flow and filtration in such states. During control conditions and hemorrhagic hypotension, with a low hematocrit (Hct) we have previously found a homogeneous cortical blood flow (Aukland et al. 1977). However, during dehydration and endotoxin shock, cortical blood flow changed to a pattern of patchy intermittent flow variations when arterial blood pressure (AP) and renal blood flow were reduced and Hct was raised above 50-55% (Kirkebø and Sebbott 1977, 1980). This phenomenon was observed irregularly and often disappeared at very low AP and renal blood flow. These two models therefore are not suitable for investigating possible mechanisms that could explain the sudden shifts in renal blood flow.

It is known that release of tourniquets after bilateral hindlimb occlusion causes a state of circulatory shock (Stoner 1961) in which AP and renal blood flow are reduced concomitantly with a rise in Hct (Takács et al. 1962). Trueta et al. (1947) also have described patchy ischemic necrosis of outer renal cortex and postulated a redistribution of renal blood flow during tourniquet shock. We therefore decided to study the local and total renal blood flow during and after total occlusion of both hindlimbs in dogs, particularly to see if the previously described patchy intermittent flow is a general consequence of raised Hct and reduced AP and renal blood flow during shock.

METHODS

The effect of bilateral hindlimb tourniquets on distribution of renal cortical blood flow was studied in 15 dogs of both

Table 1 Mean arterial pressure (AP) hematocrit (Hct) total (RAF) and local (OCF ICF) renal blood flow in control at the end of the hindlimb occlusion period and after release of the tourniquets

In Series III the tourniquets were applied before start of surgery Mean \pm S.D.

| | AP (mmHg) | RAF (ml/min g) | OCF (ml/min g) | ICF (ml/min g) | Hct (%) |
|---------------------------------|---------------|-------------------|-------------------|-------------------|------------|
| Control $n=7$ | 1.5 \pm 0.2 | 3.75 \pm 0.28 | 3.63 \pm 0.61 | 3.03 \pm 0.77 | 37.2 |
| <i>End of tourniquet period</i> | | | | | |
| Series A $n=7$ | 124 \pm 17 | 3.24 \pm 0.61 | 3.06 \pm 0.77 | 2.82 \pm 0.77 | 47.7 |
| Series B $n=8$ | 130 \pm 16 | 3.52 \pm 0.65 | 4.98 \pm 0.91 | 4.59 \pm 0.60 | 46.7 |
| Series A+B $n=15$ | 177 \pm 16 | 3.38 \pm 0.69 | 3.0 \pm 0.82 | 2.70 \pm 0.67 | 43.3 |
| <i>Tourniquet off</i> | | | | | |
| 1/4 h $n=15$ | 103 \pm 17* | 28 \pm 0.86 | 1.91 \pm 0.58* | 1.82 \pm 0.51 | |
| 1 h $n=14$ | 91 \pm 18* | 1.75 \pm 1.16 | 1.56 \pm 0.79* | 1.54 \pm 0.38* | |
| 2 h $n=14$ | 97 \pm 2 | 1.63 \pm 1.33 | 1.17 \pm 0.47* | 1.23 \pm 0.57* | |

Significantly lower than control $P < 0.05$

sexes weighing 15–30 kg. The dogs had free access to drinking water but food was restricted 18 h before surgery. Anesthesia was induced by i.v. Nembutal 3.5 mg/kg b.wt. and additional doses given when needed. The dogs were intubated with a cuffed endotracheal tube and breathed spontaneously.

The kidney was exposed retroperitoneally through a flank incision and gently dissected free. The renal artery was cannulated by a polyvinyl catheter with the tip directed upstream for injections of hydrogen saturated saline. Polyethylene catheters were put into the brachial vein and artery for infusions and AP recording respectively. The AP was measured with a Hewlett Packard transducer and recorded on a Hewlett Packard recorder model 7796A. Total renal blood flow (RAF) was measured by an electromagnetic flowprobe (Nycotron) on the renal artery and the flowmeter calibrated on a femoral artery.

L shaped platinum electrodes with 0.5 mm diameter (sensitive cone tip diameter 0.5 to 0.1 mm and 1 mm length) were inserted in the renal cortex so that 3 electrodes had the sensitive tip in the outer half and 3 had the tip in the inner half of cortex. The electrode shafts were sutured to the renal capsule. The electrodes were polarized with +0.5 V against an Ag/AgCl reference electrode placed subcutaneously on the hip. Hydrogen saturated saline with a temperature of 37°C was injected in the renal artery until a stable current was obtained on all electrodes. Then the injection was abruptly stopped and the washout curve recorded on a 8 channel Rikadenki recorder (Åstrand et al 1973). The monoexponential washout curves were plotted on a semilogarithmic paper and the flow rates were calculated from the formula $F = -\lambda \ln 0.5/T_{1/2}$ ml/min g where $T_{1/2}$ is the half life of hydrogen concentration in tissue and the tissue/blood partition coefficient λ is 1.0.

Experimental procedure

In 7 of the animals the surgery and the measurements of control blood flow were completed before the tourniquet were applied. Series A.

In 8 experiments the tourniquets were applied before the surgery started. Series B.

The wire tourniquets were placed on both hind limbs near the hip as possible and strongly tightened to stop blood flow. The occlusion lasted for 4 h. The RAF and local cortical flow were recorded continuously while measurements of local cortical flow were repeated each 20–30 min during the tourniquet period. The two tourniquets were released with <15 min interval and local cortical measurements repeated each 5–15 min for at least 1 h after tourniquet release (except in dogs). Since renal arterial saline infusion in the renal artery would tend to dilute Hct hydrogen gas was administered directly into the tracheal tube at RAF below 50% of control values. Comparison of locally injected and inhaled H_2 gases were showing no discrepancies at flow rates of 2 ml/min g less.

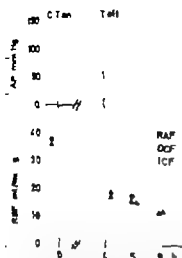
Hct was measured each hour and the total plasma proteins measured by the amino black method (Plum et al 1955) in the control period during occlusion and several times after tourniquet release.

In 5 dogs showing patchy intermittent cortical necrosis during development of shock phenolamine (10 mg b.wt) or phenoxybenzamine (ca. 3 mg/kg b.wt) was given in the renal artery to test whether the changes were permanent or disappeared. Similarly heparin (500 U) was given in 5 dogs.

At the end of each experiment the kidney was removed for 2 min and weighed. The drained kidney was used for calculations of RAF in ml/min g. The position of each electrode tip was carefully examined. Statistical evaluations were performed with Student's t -test.

RESULTS

In 7 experiments (Series A) control renal blood flow was measured about 15 min prior to application of the tourniquet. The results are given in Table 1. Local blood flow in outer cortex (OCF) and inner cortex (ICF) were 3.63 ± 0.61 and 3.03 ± 0.77 ml/min g respectively as determined from 2 control



Effect of hindlimb occlusion on renal arterial pressure and renal blood flow (RBF) in control period (C) placed tourniquet (T on to T off) and after release (T off).

Washout curves obtained with about 5 min intervals in each dog. Hct averaged $37 \pm 2\%$ and total plasma averaged 5.24 ± 0.95 g/100 ml. In the control period all cortical washout curves were monoexponential down to at least 10% of initial saturation and abrupt changes in flow were never seen before tourniquet application.

Changes during hindlimb occlusion

The wires around the hindlimb were tight in the wires around the hindlimb were tight the dogs started to hyperventilate and the AP increased although adequate anaesthesia was maintained as estimated by abolished eye lid and limb reflexes. Within 30 min the AP rose 17% (to 146 mmHg) as an average for the 7 dogs and the renal arterial blood flow (RAF) decreased 15% indicating that the renal vascular resistance increased about 15% most likely mediated by sympathetic nervous system. Ventilation and heart rate returned in about one hour but RAF returned to control level during the 4 h tourniquet period.

Within 30 min before release of the tourniquet the values were slightly reduced as shown in Table 1. The average AP was unchanged from control, whereas Hct had increased by nearly 30%. In other experiments (Series B) the tourniquets were applied before starting surgery and the first measurements in this series were performed the last

half hour before tourniquet release showing blood flow and AP nearly identical with the values given for Series A. Because of this and since the experimental procedures after tourniquet release were also identical for the two series the following results for both groups are described in common as shown in Fig. 1 and Table 1.

During the tourniquet period local cortical blood flow measured with H_2 clearance sometimes showed striking irregular patterns. The washout curves from single electrode sampling sites incidentally showed abrupt changes to a lower or a higher clearance rate. Fig. 2 shows H_2 washout curves recorded simultaneously from 4 different sampling zones in different cortical depths in one kidney. The sampling site represented by curve A showed normal saturation of hydrogen but during washout sudden shifts in rate were observed (marked with arrows). Each of these different rates were linear during replotting and allowed calculation of flow. The curve B representing another area in the cortex, showed a steady slow increase in zero-line before H_2 administration, and for some time during the H_2 injection. Then suddenly the area started to take up H_2 at normal rate (marked with arrow) and later showed a monoexponential washout curve. The interpretation must be that the area started with a blood flow close to zero and that the blood flow abruptly increased and remained high during the washout period.

During the tourniquet period such abrupt changes in the H_2 -washout curves were observed in all except 3 animals, but in 7 dogs only on one electrode. The total frequency of abrupt changes was slowly increasing in this period, but sudden shifts in flow were seen also in 4 dogs shortly after the application of the tourniquet, before Hct had increased. Though sudden local flow changes could be observed on several electrode sites, concomitant sudden changes in RAF were not detected.

A rise in Hct by 25% indicates an appreciable loss of fluid from plasma. Edema was observed on the occluded limbs in only two dogs in the tourniquet period. During the whole tourniquet period the hindlimb temperature decreased in all dogs approaching the room temperature of 22°C.

Measurements shortly after release of the tourniquets

When the tourniquets were released from both hindlimbs after 4 h of occlusion all dogs started

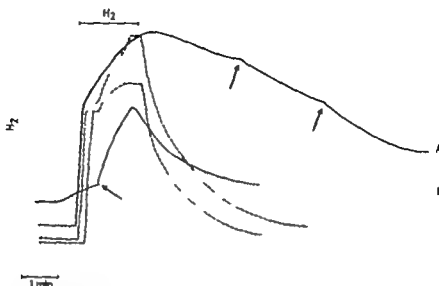


Fig. 2. H_2 washout curves of 4 single electrodes in one dog where the hindlimbs were occluded 10 min previous to the H_2 administration. Two of the curves show saddle bends (arrows). See text.

to hyperventilate and the AP began to fall. One dog died from heart arrest 25 min after release of the slings. In the period between release and heart arrest in this particular dog, the local cortical blood flow was patchy and RAF and AP steadily decreasing. Hct increased to 68% within 20 min after tourniquet release, indicating rapid fluid loss from circulated blood vessels.

All other dogs (14) lived for at least 3 hours, and the experiments were continued up to 7 hours after tourniquet release. In all dogs pronounced edema was seen in the hindlimbs.

Measurements during the first half hour after tourniquet was released showed an abrupt fall in RAF, OCF and ICF to 61, 53 and 60% of control while AP fell to 87%, all significantly lower than control (Table 1, Fig. 1). Renal vascular resistance was calculated to be about 35% higher than control but about the same as shortly after tourniquet occlusion.

Local blood flow heterogeneity

In 7 dogs abrupt changes in washout rate of H_2 shortly after release of tourniquets (as in Fig.

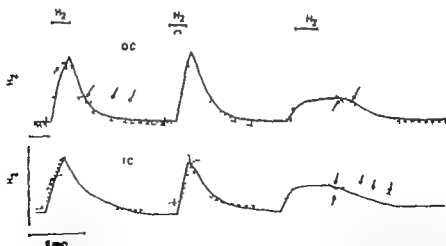


Fig. 3A. H_2 washout curves of 3 single electrodes in outer (OC) and 3 in inner (IC) cortex of a dog. The first H_2 administration was made about 5 min after tourniquet release. The curves show independent sudden changes in washout rate (arrows) both in IC and OC (see text).



Fig 3B H_t washout curves of electrodes in outer (OC) and inner (IC) cortex. The H_t saturation started about 32 min after tourniquet release. When saline was infused the washout started at different time on different electrodes (arrows) (see text).

three consecutive washout curves for 6 electrodes in one dog are shown. The washout curves recorded 5 min after release of the slings of the curves showed constant flow while the curve in the upper panel described 4 min flow rates. Thus sampling area started at a rate of 2.18 min fell abruptly to at the first arrow then started a slow washout arrow) (ca. 0.30 min) before it again at the second arrow increased the washout rate to 1.30 min. Each single component of the washout curve is monoexponential.

The next series of washout curves showed well preserved blood flow and fairly homogeneous blood flow in all sampling areas whereas the third washout curve showed an inhomogeneous flow pattern over flow in all tested areas and as well a lower flow rate. The solid curve in the upper panel starts with a low rate, but at the arrow suddenly increased its rate. In the lower panel the broken line starts with a fairly well preserved flow (0.94 ml/min) changes to zero then regains the initial flow after 30 min before a second period with flow until finally the hydrogen gas is washed out at a rate of 1.03 min.

The best washout curves shown in panel 3B were recorded 32 min after release of the tourniquets, and showed slow saturation of H_t in all areas. One area (marked with solid line bottom panel) was probably saturated only by diffusion from neighbour areas with higher H_t concentration and continued a slow saturation for long period. The washout rates in all sampling sites were low until i.e. infusion of saline was started (20 ml/min). After a few minutes the washout rates increased at different

sampling sites at different time as marked with arrows.

Correspondingly the electromagnetically measured flow was also close to zero and increased concomitant with local flow when saline was infused. When infusion started Hct was 68% indicating a fast severe plasma fluid loss after release of tourniquet.

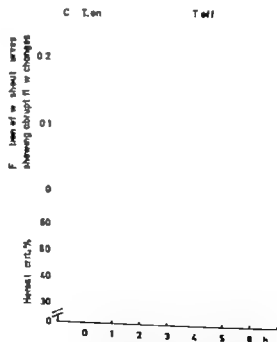


Fig 4 Average hematocrit (below) and fraction of single washout curves within each hour showing abrupt flow changes (above) in control (C) after application (T on) and after release (T off) of the tourniquets.

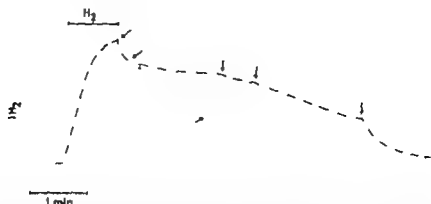


Fig. 3B Washout from 2 single electrodes about 60 min after phenoxylbenzamine administration showing several changes in washout rate (arrows)

Patchy intermittent flow changes were totally observed in all except 1 animal in 7 dogs only 7–15 changes on 1–2 electrodes, but in 7 dogs 15–45 times irregularly from different sampling sites. The frequency increased markedly after release of the tourniquets. The average fractions of washout curves showing abrupt flow shifts within each hour were about 0.7 in the 2nd and 3rd h after tourniquet release (Fig. 4).

Measurements 2 h after release of the tourniquets

AP, RAIP and local cortical blood flow continued to fall throughout the whole experimental period so that 2 h after tourniquet release the AP, RAIP, OCI and ICF were reduced to 74, 43, 32 and 41% of control respectively (Fig. 1). OCI 1.17 ± 0.47 ml/min/g was not significantly different from ICF 1.23 ± 0.57 ml/min/g.

The total plasma protein concentration increased to 6.32 ± 1.1 mg/100 ml plasma, i.e. 21% above control, while Hct increased to $58 \pm 5\%$, i.e. 57% above control. This shows that the plasma fluid loss was accompanied with leakage of plasma protein.

Abrupt shifts in rate of washout were still observed after more than 2 h, but less frequently as blood flow decreased to very low levels. Before ending 5 experiments the dogs were given 0–0.8 l of saline. 1 dog even within the 1 h period after tourniquet release (Fig. 3B) to restore the AP and blood flow. Saline infusion increased renal blood flow rapidly.

In 5 experiments the α receptors in the renal vascular bed were blocked by phentolamine or phenoxylbenzamine at a time with frequent shifts in local cortical flow on one or more electrodes. Complete block was tested with 1 μ l injections of norad-

renaline. This block did not prevent the appearance of sudden changes in local flow, as shown in Fig. 1, where one washout curve has 4 different washout rates and the other 2 rates close to one lower rate. Treatment RAIP increased slightly after a repeat blocking agent.

Further heparinization of 3 dogs did not prevent appearance of vascular shifts, suggesting that microthrombosis cannot be the explanation of the intermittent flow.

DISCUSSION

The local cortical blood flow obtained in control was well within normal range previously obtained with the hydrogen washout method (Aukland et al. 1977; Tyssebo & Kirkebo 1979) and well correlated with the total renal blood flow measured with electromagnetic flowmeter.

Sympathetic nerve activity after tourniquet application

Measurements performed few min after the occlusion showed transitory hyperventilation, increased AP and slightly decreased renal perfusion. This is compatible with an increased nervous tone in the renal vascular resistance. Trueta et al. (1950) described a reduction of the renal artery flow immediately after tourniquet application in rabbits, concluding that this vasoconstriction was caused by neurovascular reflexes initiated in the injured limb. This agrees with the findings of Barnes & Trenchard (1947) that a tourniquet applied to one limb can cause a vascular spasm in the contralateral limb, and that denervation prevented the changes. Rowell (1979) concluded recently that the response was mediated by α fibers from the occluded man.

d on angiographic studies. Trueta et al. also described diversion of intrarenal blood from superficial to juxtamedullary glomerular application of a tourniquet or by stimulation of splanchnic nerves.

Similar redistribution was reported by Ariz et al. (1968) using ^{86}Kr clearance during lumbic nerve stimulation or ligation of the renal arteries. On the contrary in the present study we found that OCF and ICF were reduced in the same proportion. Likewise bilateral occlusion of carotid arteries in conscious dogs increased vascular resistance by about 40% thus inducing some reflex sympathetic activity but the OCF/ICF ratio remained unchanged (Kirkebo & Tysebotn 1974). However Aukland (1976) has demonstrated that multicomponent analysis of the clearance curves gives unrealistic zonal flow and volume fractions.

In local cortical blood flow often changed from homogeneous flow pattern to an uneven and patchy distribution of cortical blood flow. We previously observed a similar pattern when Hct had increased to above 55% during dehydration (Kirkebo & Tysebotn 1977) or in endotoxaemia (Kirkebo & Tysebotn, 1980). In the present study the abrupt shifts were observed shortly after tourniquet application when the Hct could hardly have increased to high levels, since Hct at the end of 4 h of occlusion only had reached 47%. Increased sympathetic nerve activity could possibly increase Hct by a reflexory contraction of the spleen, pushing packed red cells into the circulation. Since 150 ml red cells would be sufficient to account for the observed rise in Hct during occlusion.

However at the same time the increased release of ketone and/or increased catecholamine blood levels could draw fluid from the unaffected muscles thereby tend to reduce Hct (Mellander & Jonasson, 1968).

Even though the slings around the limbs were tightened as firmly as possible some blood may have passed through the arteries into the distal limbs. On the other hand, the hindlimbs are not easily seen to swell until release of the slings, so that only negligible amount of blood could have passed behind the slings during the 4 h occlusion and did not cause the generally observed increase of Hct.

The patchy intermittent flow in the early phase may have been mediated by nervous reflexes con-

comitant with hyperventilation and a rise in AP. An explanation based on circulated vasoactive hormones is not probable considering the prompt occurrence after tourniquet application and that infusion of vasoactive substances in the renal artery caused a homogeneous change in cortical blood flow (Tysebotn & Kirkebo 1975, 1979).

However when the tourniquet was tightened some tissue on the proximal side may be damaged either by direct trauma or by ischemia. Toxic products could in time enter the general circulation eventually by leakage beyond the tourniquet or from the damaged tissue and possibly cause some change in total or local renal blood flow. Such explanations are not likely for the flow shifts during the early phase of hindlimb occlusion but cannot be ruled out during the late phase of the 4 h occlusion period.

Heterogeneous cortical blood flow after release of the tourniquet

The dogs started again to hyperventilate immediately after release of the tourniquet, probably due to acidotoxic metabolites entering the systemic circulation from the cooled and damaged hindlimb tissue. This explanation is supported by Liu (1971) who found the same responses in untraumatized dogs when cross circulating blood from dogs traumatized with hindlimb occlusion of comparable duration to that in our animals. These released substances lead to an abnormal leakage of fluid from the capillary bed causing edema and Hct elevation. As the occluded legs again were circulated the hindlimbs started to swell profusely.

Takács & Kallós (1957) found the same changes in blood flow whether denervating the kidney or not, concluding that the circulating agents caused a vasoconstriction. With a Hct of 58% as in the present study it is doubtful whether the renal vascular hindrance was increased, since the viscosity rise is probably greater than the calculated increase in resistance (Schmid-Schönbein 1976). However the vascular responses in outer and inner cortex were equal.

At high Hct and decreased AP the frequency of intermittent patchy flow distribution increased as previously described (Kirkebo & Tysebotn 1977, 1980). However since patchy intermittent flow also was seen before Hct was increased, high Hct cannot be the only explanation for patchy flow but most likely facilitates the observed changes.

During development of shock the plasma leakage continued and the total renal perfusion could eventually cease (Fig 3B). However the situation was reversible: dilution of the red cells by saline infusion reopened the vessels and both total and local flow increased rapidly after few min of infusion. This must mean that permanent thrombi were not formed at this state, indicating that local clotting of blood in limited areas is not a likely explanation. This is further supported by the findings that heparinization with adequate doses did not prevent the occurrence of patchy flow changes.

Hansson (1965) has shown that the kidney is vulnerable to thrombocyte aggregation induced by administration of crushed connective tissue suspension or ADP. Starting with the same procedure Jorgensen et al (1970) observed later patchy tubular necrosis and focal glomerular nephritis. On the other hand Collan & Alho (1973) found no microthromboses of platelets by ultramicroscopy until 74 h after opening of tourniquets.

An uneven distribution and affinity of α receptors responding to nerve activity or circulating catecholamines could possibly explain the patchy cortical flow, but since the α blocking agents did not reduce the occurrence of blood flow shifts, this could not be the only reason. One may suspect that local agents might play a role in provoking the phenomenon.

In conclusion OCF and ICF were proportionally reduced during tourniquet shock. The occurrence of patchy intermittent cortical ischemia is probably facilitated by a high Hct during shock, but was also seen before Hct did rise: α receptor blocking or heparinization did not abolish the phenomenon.

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Effects of glucagon and pentagastrin on glibenclamide-induced insulin release

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It was recently reported that administration of sulphonylureas may lead to stimulation of the release of glucagon and gastrin-like peptide. These peptides may then eventually influence the insulin releasing action of the sulphonylureas. Therefore the effects of glucagon and pentagastrin on basal and glibenclamide-induced insulin secretion were studied. It was found that at a dose of 4.25 $\mu\text{mol/kg}$ the two peptides induced a significant stimulation of basal insulin secretion. However, pentagastrin at the dose of 4.25 $\mu\text{mol/kg}$ did not influence the insulin release induced by glibenclamide, whereas an equimolar dose of glucagon potentiated the insulin secretory response to this sulphonylurea drug by about 40%. Glucagon in contrast to pentagastrin thus positively modulates the insulin secretory pathway stimulated by the sulphonylurea drug glibenclamide.

Key words. Glucagon, pentagastrin, glibenclamide, insulin secretion.

Sulphonylureas used in the treatment of insulin-dependent diabetes have been shown to stimulate the release of insulin (Loubatieres 1969). Also the release of other hormones such as glucagon and gastrin may be influenced by these drugs. The release of insulin has been reported to be stimulated by sulphonylureas (Loubatieres et al. 1974) inhibited (Samols, Tyler & Loubatieres 1969) or unaltered (Kajimura, Katsura & Loubatieres 1974) by sulphonylureas. Later it was shown that in the rat tolbutamide in high concentrations stimulates glucagon release whereas in low concentrations it stimulates glucagon release (Grodsky et al. 1977). Recently it was reported that in the cat sulphonylureas also stimulate the release of immunoreactive gastrin of antral as well as of duodenal origin (Uvås-Waltesen et al. 1979). Sulphonylureas stimulate the release of glucagon and if the reported immunoreactive gastrin released by sulphonylureas shares biological activity with authentic gastrin the effects of glucagon and gastrin on sulphonylurea-induced insulin release might be of importance. In the present study the *in vivo* effects of glucagon and pentagastrin on insulin release stimulated by the sulphonylurea drug glibenclamide were investigated in mice.

METHODS

Drugs

Pure porcine glucagon was obtained from Novo Research Institute, Copenhagen, Denmark. Pentagastrin (Peptavlon®) was from ICI Ltd, Macclesfield, Cheshire, Great Britain and glibenclamide from Boehringer Mannheim GmbH, Germany. The peptides were dissolved in saline with addition of 0.1% gelatine.

Animals

Female mice of the NMRI strain (Laboratory animal Breeding, Län, Denmark) weighing 25-35 g, were used. The animals were fed standard pellets (Astra-Ewos, Södertälje, Sweden), and tap water *ad libitum* before and throughout the experiments.

Experiments

1. Effects of glucagon or pentagastrin on basal insulin release. Each of the 2 peptides was injected in tail vein, 4.25 $\mu\text{mol/kg}$, 10 $\mu\text{l/g}$ b.wt. was injected. Control animals were injected with saline. Two minutes after the injection 250 μl blood was drawn by orbital puncture. The mice were anaesthetized throughout the experiments.

2. Effects of glucagon or pentagastrin on glibenclamide-induced insulin secretion. Each of the peptides was injected 5 $\mu\text{l/g}$, 15 min prior to rapid injection of 5 $\mu\text{l/g}$ glibenclamide. Controls were injected with saline and glibenclamide, or with saline alone, then after the last injection blood was collected. This sequence of the injections was chosen in order to mimic the anticipated glibenclamide-induced hormonal endocrine effect.

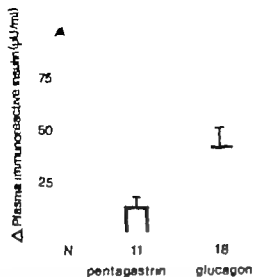


Fig. 1 Increase in plasma immunoreactive insulin following the intravenous injection of 4.25 nmol/kg body weight of pentagastrin and glucagon respectively. Blood sampling was performed 2 min after the injection. Bars indicate standard errors of the mean. N =number of mice.

fects since the peptides released by oral intake of sulphonylureas may reach the insulin cells before the drug. Repeated experiments in this laboratory have shown that the maximal concentration of immunoreactive insulin in mouse plasma following a rapid i.v. injection of glibenclamide is achieved after 1.5–5 min.

Glibenclamide was administered in a dose giving about 50% of its maximal insulin response. This dose was 62.5 μg/kg. Glucagon and pentagastrin were administered at a dose of 4.25 nmol/kg.

Determination of insulin

The concentrations of immunoreactive insulin (IRI) in plasma were determined by a radioimmunoassay technique (Hedjog 1966) using 125 I-labelled pig insulin and guinea pig anti-pig insulin.

RESULTS

Effects of glucagon or pentagastrin on basal insulin release

Each of the 2 peptides was injected i.v. 4.25 nmol/kg and 7 min later blood was collected. Control animals were given saline. It was found (Fig. 1) that glucagon enhanced the plasma IRI concentrations by 42.3 ± 9.5 μU/ml above the controls ($P < 0.001$), whereas pentagastrin induced an elevation of the plasma IRI concentrations by 13.2 ± 3.9 μU/ml ($P < 0.01$).

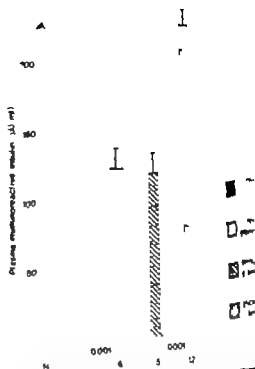


Fig. 2 Effect of pentagastrin and glucagon, respectively, on insulin release stimulated by glibenclamide 62.5 μg/kg (open column), pentagastrin (4.25 nmol/kg) (stippled column) or glucagon (4.25 nmol/kg) (stippled column) injected intravenously 15 s prior to glibenclamide 62.5 μg/kg. Blood sampling was performed 2 min after glibenclamide injection. Control animals received saline (black column). Bars indicate standard errors of the mean. P =probability level of random difference as determined by the t -test. N =number of mice.

Effects of glucagon or pentagastrin on glibenclamide-induced insulin release

Glibenclamide 62.5 μg/kg injected i.v. lowered the plasma IRI concentrations to 124 ± 15.4 μU/ml ($P < 0.001$ vs. the saline injected controls) (Fig. 2). Glucagon 4.25 nmol/kg was injected 15 s prior to glibenclamide and it was found that the plasma IRI concentrations were further increased to 126.2 ± 11.4 μU/ml ($P < 0.001$ vs. the glibenclamide control group). Since glucagon exerted a stimulatory effect on basal insulin secretion by itself, the expected concentrations of plasma IRI following the injections of glucagon plus glibenclamide can be calculated to be about 160 μU/ml. The observed plasma IRI concentrations in the group treated with glucagon plus glibenclamide 126.2 ± 11.4 μU/ml are significantly higher than this expected ($P < 0.001$). Thus it was found that glucagon potentiated glibenclamide induced insulin release by about 40%.

tion of pentagastrin, 4.25 nmol/kg plus benide resulted in a mean plasma IRI con- of 119.6 \pm 15.2 μ U/ml (n.s. vs the gli- side control group). Thus, contrary to gli- pentagastrin did not affect glibenclamide insulin release.

DISCUSSION

It stimulates the release of insulin (Rehfeld of Fig. 1). It may function as an intrapan- regulator of insulin release since it may be cretic neural (Larsson & Rehfeld 1979) or tic neonatal endocrine (Larsson et al. 1976). Furthermore, immunoreactive gastrin of and neural origin in the rat was recently strated to be released by glibenclamide (Uv allesten et al. 1979). If this immunoreactive possesses bioactivity gastrin might be of not in mediating insulin releasing effects of phosylurea drugs. This could be a parallel to s ported glibenclamide-induced release of a al factor with insulin releasing properties sties et al. 1977). However we failed to any influence of pentagastrin on gliben- le-induced insulin release. This does not le important effects of gastrin in mediating ctions of glibenclamide.

Glucagon has earlier been shown to stimulate the e of insulin (Samols, Marri & Marks 1965 per & Orr 1976). This study shows that ay to pentagastrin glucagon potentiated clamide-induced insulin secretion. A syner etween glucagon and tolbutamide with regard e insulin secretion in man has earlier been reported (Orri & Cerasi 1977). Such synergism might e importance in sulphonylurea treatment of ies if these drugs stimulate the release of n. Moderately increased circulating levels agone in diabetes (Unger 1978, Lefebvre & ka 1979) may also make sulphonylurea drugs onerful in stimulating the release of insulin. Glucose and sulphonylurea drugs show some eases in their insulinotropic effects. Me- on and fluxes of calcium (Malaisse Bri- s & Baird 1973, Malaisse, Pipeleers & Mahy l handling of cAMP (Grill & Cerasi 1973, Sams e 1974), lysosomal activation (Lundquist Lundquist 1975) and intracellular amines (Lundquist, Elholm & Ericson 1971, Lundquist Ericson, Håkanson & Lundquist 1977) seem

all to be of importance in a similar way for these secretagogues. Recent studies in our laboratory (unpublished) indicate that glucose-stimulated insulin release in mice is potentiated by glucagon but not by pentagastrin which further shows the similarity between glucose and glibenclamide in their insulin releasing activity. Glucagon in contrast to pentagastrin thus may positively modulate some events of importance for the insulin-releasing activity of both glucose and glibenclamide.

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Simultaneous measurements of capillary filtration and diffusion capacities during graded infusions of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) into the rat hindquarter vascular bed

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RIPPE B & FOLKOW B: Simultaneous measurements of capillary filtration and diffusion capacities during graded infusions of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) into the rat hindquarter vascular bed. *Acta Physiol Scand* 1980; 109: 265-273. Received 8 Nov 1979. ISSN 0001-6772. Department of Physiology, University of Göteborg, Sweden.

The relationships between capillary diffusion capacity (PS) for Cr-EDTA, respective capillary filtration capacity (CFC) and vascular resistance during graded intraarterial infusions of NA and 5-HT into the artificially constant flow perfused rat hindquarter vascular bed were investigated. During maximal vasodilatation PS for Cr-EDTA was some 5.5-5.7 ml/min \times 100 g, CFC some 0.04 ml/min \times 100 g, while vascular resistance was 2.8 mmHg \times ml⁻¹ \times min⁻¹ \times 100 g (PRU₁₀₀) and isogravimetric capillary pressure 12.8 mmHg on an average. Setting out from maximal vasodilatation, successive doses of NA and 5-HT produced graded reductions in capillary surface area as reflected by progressive decreases in both PS for Cr-EDTA and CFC. These changes occurred simultaneously with progressive increases in both pre- and postcapillary resistances, causing elevations in both arterial and capillary hydrostatic pressures and hence in capillary fluid filtration at constant flow. Capillary hydrostatic pressure increased maximally to 45 mmHg (calculated for NA) and vascular resistance to some 21 mmHg \times ml⁻¹ \times min⁻¹ \times 100 g on an average. PS for Cr-EDTA decreased maximally to some 0.7 ml/min \times 100 g for both NA and 5-HT and furthermore the relationships between PS for Cr-EDTA and PRU₁₀₀ for NA respective 5-HT were almost identical. This was taken to indicate that capillary surface area for substance exchange is affected similarly by both drugs. However, the CFC-PRU₁₀₀ relationship was shifted towards some 30-50% higher CFC values for 5-HT than for NA at almost every level of vasoconstriction. This might suggest that 5-HT besides reducing capillary surface area also induces moderate increases in capillary permeability through increases in number and/or radius of large pores (gaps) (cf. Rippe, Kuriya & Folkow 1978). Even during NA-induced vasoconstriction, when virtually no changes in capillary permeability occurred, PS for Cr-EDTA was reduced to a relatively greater extent than CFC, the discrepancy being most pronounced during marked vasoconstriction. The significance of this finding is discussed.

Key words: Capillary permeability, indicator diffusion, capillary filtration, isogravimetric capillary pressure, rat, noradrenaline, 5-HT.

earlier study (Rippe, Kuriya & Folkow 1978) capillary filtration and diffusion capacities were followed simultaneously in the maximally dilated rat hindquarter vascular bed over a wide range of induced changes in capillary permeability-surface product (PS). This study included a ramification of capillary network by means of i.a. injected cytochromes (Z=15 μ m) to create a stable model situation for the in vivo events when precapillary or muscle constriction reduces the perfused

capillary surface area ('precapillary sphincter function'). The diffusion capacity (PS) for Cr-EDTA, measured with a colorimetric on-line variant of the single injection indicator diffusion method (Rippe & Stage 1978) then decreased largely in proportion to the increased resistance to flow after microvascular plugging, while capillary filtration coefficient (CFC) decreased less. It was concluded that the induced type of precapillary plugging implied a capillary flow pattern that ranged from complete

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74) were performed. Isogravimetric capillary pressure (P_c) is determined as described earlier (Ejlertsen et al. 1974; Kamaya, Rippe & Folkow 1979). NA or 5-HT was infused in increasing concentrations in 5 to 6 steps until maximal vasoconstriction was reached as indicated by maximal P_c values. Care was taken to make the time periods as short as possible, every step being of ~ 4 min duration in order to avoid too much of edema formation as the preparation, as a result of the drug-induced postcapillary vasoconstriction which corresponded to a raised P_c in the constant-flow situation. During every change in CFC, P_c and capillary field equilibrium were recorded and double determinations of CFC were performed as far as possible.

In 5 experiments NA and 5-HT were given consecutively in the same preparation, the sequence between them being randomized. Increasing doses of either NA or 5-HT were given in 3 pilot experiments. In an additional series of 4 experiments maximal doses of NA was administered immediately after the control period.

Calculations were performed as described earlier (Rippe et al. 1977; Rippe, Kamaya & Folkow 1978). The P_c was calculated according to the formula

$$P_c = (Q - U) / (2J) \quad (1)$$
 where Q is the directly measured flow and J the filtration rate while E is the reflection coefficient. Arterial concentrations (C_a) up to the three highest concentrations had decreased to around 50% of peak values as used for the calculations. In the present study the convective contribution to total solute transport was slightly underestimated. The reason is that no attempt was made for transcapillary losses of the reference markers in the present study. At filtration rate of 0.3 ml/100 g the convective contribution to total transport has been determined to 5–6% (Rippe, Kamaya & Folkow 1974) of total transport which means that total transport was underestimated by some 3–5%.

RESULTS

4 and 5-HT effects on pre- and postcapillary resistances

Before control, before the start of the drug infusion, P_c was 37.5 ± 0.5 mmHg (mean \pm S.E.), P was 0.4 mmHg, perfusate flow (Q) 1.3 ± 0.3 ml/min/100 g and PRU_{pre} 1.8 ± 0.1 mmHg \times min \times ml $^{-1}$ \times g $^{-1}$ for the whole material. Isogravimetric capillary pressure (P_c) during control was estimated to be 37.5 mmHg which agrees with earlier P_c determinations on the preparation during dextran-Tyrod perfusion (Kamaya, Rippe and Folkow 1979). The relation between pre- and postcapillary resistance during control was in this study around 1/10 which is slightly higher than during pure dextran perfusion (around 1/11 Folkow et al. 1977). The reason may partly be that the precapillary smooth muscle cells are more difficult to keep fully

relaxed when horse serum is present in the dextran perfusate partly that capillary permeability characteristics are slightly different as reflected by a higher CFC value (around 0.035) and a lower P_c (around 1 mmHg) when dextran Tyrode is used.

As flow was kept constant throughout each experiment and also relatively unchanged from experiment to experiment (11.5 – 14.0 ml/min \times 100 g) changes in (P_c , P) during drug infusion closely reflect changes in total resistance to flow. In Fig. 1 (P_c , P) values are plotted against the dose of NA or 5-HT. The dose-response curves are almost identical to those obtained in earlier studies from this group (e.g. Folkow et al. 1977; Åkhrind et al. 1977). During every dose step the rate of capillary filtration was recorded as well.

In Fig. 2 filtration rate is plotted against PRU_{pre} . Provided that capillary permeability and tissue hydrostatic and colloid osmotic pressures remain essentially constant the drug-induced P_c increases (above P_c) can be assessed by dividing the filtration rate by the prevailing CFC value (Folkow et al. 1974; Ejlertsen et al. 1974). As it could be suspected that 5-HT affects capillary permeability as well (see below) P_c determinations and hence determinations of pre- and postcapillary resistances were performed only on the NA data. Such an analysis is shown in Fig. 3. During NA induced maximal vasoconstriction P_c increased to 57.3 ± 5.3 mmHg and P to 41.4 ± 2.4 mmHg, while P was kept constant at 0.8 ± 0.4 mmHg. As mentioned above P_c during maximal vasoconstriction was obtained under the assumptions that capillary permeability and tissue hydrostatic and osmotic pressures were largely unchanged during the course of the drug infusion. However adding 5–10 ml of fluid to the interstitial space which was the consequence of the drug-induced capillary filtration up to the level of maximal vasoconstriction, usually creates around 3 mmHg of absorptive forces at the capillary level (Ejlertsen et al. 1974). This was also evident from 4 experiments in which supramaximal NA doses were given directly after the control period when filtration rates were some 10–15% higher than in Fig. 2 at maximal vasoconstriction. Thus, a P_c value around 45 mmHg seems more appropriate than the value given above. The corrected pre- to postcapillary resistance ratio at maximal vasoconstriction is then around 5/1 which closely agrees with the value given by Folkow et al. 1977. The moderately higher P_c and P values obtained in

flow cessation in some capillaries via various degrees of reduced flow in capillaries fed by collaterals up to a situation of unimpeded flow in nearly all capillaries. In such a situation PS should be more reduced than CFC since it is more affected by flow limitation and heterogeneity than is the filtration process.

Towards this background it was considered of importance also to investigate the impact on PS for Cr EDTA CFC and capillary fluid equilibrium of graded vasoconstrictions induced by infusion of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) which in the prevailing situation of constant flow perfusion ought to produce a sufficiently well maintained pattern of microvascular constriction to allow for repeated measurements. Such an investigation is of particular interest since existing data in this field show considerable disagreement (cf Haddy, Scott & Grega 1976). Thus infusion of noradrenaline at constant flow perfusion of dog skeletal muscle has been reported to either decrease ^{86}Rb uptake (Szwed & Friedman 1975) or to increase it (Gable et al 1964) while CFC appeared to remain largely at control after a transient decrease during similar conditions (Kaiser & Diana 1974). In contrast during in vivo constant pressure conditions CFC is reported to increase beyond resting control during noradrenaline infusion (e.g. Järhult 1971) while ^{86}Rb uptake is reported to decrease (Appelgren & Lewis 1968). However in none of the mentioned studies CFC and PS were measured simultaneously and it is therefore impossible to judge whether the apparent discrepancies between the changes in CFC and PS are due to real differences between the two capillary exchange parameters or simply reflect methodological differences and/or different patterns of vascular responses due to the prevailing experimental situation.

Also concerning the effect of 5-hydroxytryptamine (5-HT) on transcapillary exchange processes there are some controversies, perhaps also due to the fact that 5-HT effects on capillary permeability might be species dependent (cf Haddy, Scott & Grega 1976). Thus there is evidence that 5-HT increases capillary permeability in rat (e.g. Rowley & Benditt 1946; Majno, Palade & Shoefel 1961; Gabbiani, Badonnel & Majno 1970) while it seems to be largely unaffected in dog (e.g. Merrill et al 1974) rabbit (Ebert & Graham 1966) and in man (Glover et al 1958; Roddie, Shephard & Whelan 1955).

In the present experiments the effects of graded

infusions of NA and 5-HT on PS for Cr EDTA CFC and capillary fluid equilibrium were studied under the initially maximally vasodilated vascular bed of rat hindquarters during well controlled hydrodynamic conditions and at constant flow rate. Studies in which capillary diffusion and PS capacities were measured simultaneously (Rippe, Kamuya & Folkow 1978; Rippe & Grön 1978) indicate that such an approach can provide detailed information about drug induced changes in capillary permeability-surface area product.

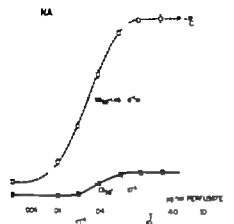
METHODS

Experiments were undertaken on the isolated hindquarters from 16 male Wistar albino rats weighing between 250 and 380 g. The preparations were arranged for the gravimetric technique (Poppenheimer & Soto 1959) and for a colorimetric on-line variant of the perfusion method (Rippe & Stage 1978).

The preparation as well as the general incubation arrangements have been described earlier (Rippe & Folkow 1978). Briefly the hindquarters were isolated by tight mass-ligatures at a level between the renal and lumbar vessels and the spinal channel and bone marrow were plugged with cotton wool soaked in saline. Leakage from vessels or tissue was accepted. Tail veins were excluded by ligatures so that about 4% consisted of skeletal muscle, the remainder being adipose tissue and bone.

The preparation was artificially perfused at constant flow via the aorta by means of Harvard perfusion pump. Arterial pressure (P_a) was measured continuously in the tail artery and venous outflow pressure (P_v) in one of the renal veins. The completely isolated hindquarters was continuously weighed on a balance plate connected to a strain gauge and tissue weight as well as pressures were recorded on a Grass polygraph. The venous outflow and draining the caval vein was coupled in series with a meter and a densitometer system for simultaneous readings of Cr EDTA and Cardio-Green (brominated oil). These indicators were used for measurements of Cr EDTA diffusion capacity (Rippe & Stage 1978), pH and flow curves were monitored on a potentiometer (Rika Denki model B34). The free end of the venous outflow cannula could be adjusted to any desired level to set the venous outflow pressure. P_v flow and P_a were controlled by adjusting the setting of the perfusion pump. 4 per cent dextran (Macrodex, M.W. about 40,000, AB Pharmacia, Sweden) in Tyrode solution was the perfusate to which was added horse serum (SBL Serum SBL, Sweden) 100 ml/l. The perfusate was perfused with a mixture of 97% O₂ and 3% CO₂ and kept at 37°C. Flow was kept constant at 11.5–14 (mean 13.3) ml/100 g throughout the experiment. During control experiments vasodilatation was induced by drug injections (0.1 ml) of papaverine.

In the control situation three measurements of PS for Cr EDTA (Rippe & Stage) and of CFC (Folkow



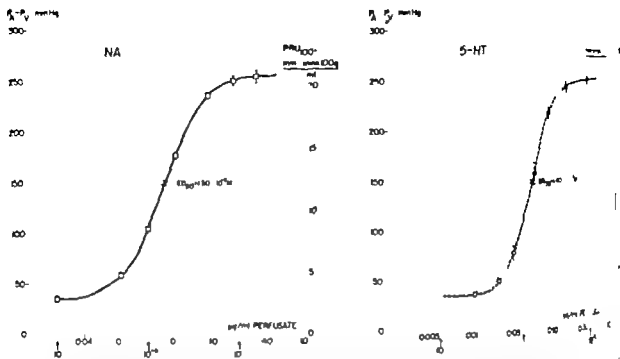


Fig 1 Average constant flow perfusion pressure plotted against concentration of NA (left part: solid line) and 5-HT (right part: broken line). Total peripheral vascular resistance expressed in PRU_{100} is also indicated in the figure (right part: broken line). Mean values \pm S.E. from 9 expts with NA (squares) and 9 with 5-HT (circles) are shown. ED_{50} denotes the concentration of either drug effective of producing 50% of maximal pressor response.

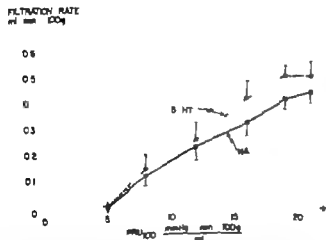


Fig 2 Filtration rate vs peripheral vascular resistance during infusion of increasing doses of NA (solid line) and 5-HT (broken line) during constant flow. The increase in capillary filtration with increasing PRU_{100} and drug concentration during constant flow is most likely due to a progressive increase in postcapillary resistance and hence in P_c (cf. Fig. 3). As regards 5-HT increases in capillary permeability producing decreases in effective transmural pressure gradient might also be taken into account in explaining the data. Though 5-HT seemed produce higher filtration rates than NA at every level of increased peripheral resistance the difference is not statistically significant.

the present study compared with that of Folkow et al. 1977 could be explained by the 10-fold higher flows.

Simultaneous changes in CFC and PS for Cr EDTA during NA-infusion

In Fig. 4 the simultaneous changes in CFC and PS for Cr EDTA during NA infusion and constant flow conditions (flow 11.5–14 ml/min \times 100 g) plotted against total vascular resistance expressed in PRU_{100} units. It is seen that the relative changes in CFC and PS closely follow each other and are reduced down to one third of controls by maximal constriction but at further PS reductions simultaneous CFC changes are relatively minor. Further the NA effects on the true resistance of vessels are initially more pronounced than the effects on precapillary sphincter vessels when expressed as reduced PS and CFC. In case the two effects were to run parallelly the relation between CFC-PS and PRU_{100} would have followed the dotted line in Fig. 4. From the state of maximal vasoconstriction PS ranged from 5.7 ml/min \times 100 g (8 times decrease) while CFC ranged from about 0.04 to 0.01 ml/min \times mmHg \times 100 g (4 times decrease).

are far more reduced than in CFC. This latter response resembles that obtained during precapillary constrictor fibre stimulation with microspheres (2–15 μ m) (Rippe & Folkow 1978) which reduces PS substantially more than CFC. This response pattern is due to the fact that substantial filtration still occurs in capillaries perfused at very low flow rates. Filtration is here severely reduced by flow limitation.

Overall these results in a well controlled constant flow situation in vitro are in agreement with results by Lundvall & Järhult (1976). In the rat hindquarter fibre stimulation to the cutaneous vascular bed they found evidence for adrenergic dilatation of precapillary sphincter segments paralleling the α -adrenergic constriction of arteriolar segments. To some extent also noted in NA infusions (cf. Järhult 1971).

Overall the observed discrepancy between CFC changes during marked NA vasoconstriction might also reflect increases of capillary permeability caused by the capillary pressure rise consequent to the constant-flow situation. However, in the rat hindquarter vascular bed pronounced increases in capillary permeability occur at capillary hydrostatic pressures well below 45–50 mmHg (Rippe, Kamiya & Folkow 1979). Such a mechanism would therefore hardly affect the measurement.

During 5-HT infusion the relationship between flow and resistance increases was almost identical to that during NA-induced vasoconstriction while the CFC reductions were displaced 30–40% upwards compared with the situation during vasoconstriction. This may reflect a different reaction pattern on the precapillary vessels for 5-HT than for NA and/or that 5-HT increases capillary permeability. At high 5-HT concentrations there is evidence of a relative decrease in small precapillary resistance in dog forelimb muscle (between small vessel resistance in the skin in the rat) (Nenill et al. 1974).

In the rat hindquarter preparation mainly consisting of skeletal muscle (1971), the possibility of a phasic dilatation in response to 5-HT paralleling the increased resistance increases cannot be completely ruled out. The main mechanism however behind relatively higher levels of CFC during 5-HT infusion is likely to be that 5-HT increases capillary permeability in rat, well documented in this respect (e.g. Rowley & Benditt 1956; Sparrow &

Wilhelm 1957; Majno & Palade 1961; Majno, Gilmore & Leventhal 1967). This permeability increase seems to reflect a formation of venular gaps (large pores) (cf. Grotte 1956) probably by means of endothelial cell contraction causing partial detachment of intercellular junctions (Majno, Gilmore & Leventhal 1967; Majno, Shea & Leventhal 1969). Further evidence for such a mechanism has recently been obtained also for histamine in various experiments (e.g. Rippe & Grega 1978).

Maximal 5-HT increases in capillary permeability were obtained at 5-HT concentrations around 0.05–0.08 μ g/ml (1.2×10^{-5} M) as indicated by the CFC data in Fig. 5 compared with those in Fig. 4. Thus on a molar basis 5-HT seems to be at least 100 times more potent than histamine which in the rat is effective in the concentration range of 3–20 μ g/ml (~ 7 – 18×10^{-5} M) (Rippe & Grega 1978) in agreement with earlier studies (Sparrow & Wilhelm 1957). On the other hand the 5-HT induced CFC increase above that during NA infusion was only some 30–40% which is 5–8 times lower than the maximal CFC changes evoked by histamine at a constant capillary surface area (cf. Rippe, Kamiya & Folkow 1978). It is in line with the comparatively weak permeability increasing effects of 5-HT reported by Majno, Gilmore & Leventhal (1967) who studied vascular permeability after local applications of bradykinin, histamine and large doses of 5-HT (100 μ g/ml) to the rat cremaster muscle. Assuming a rough proportionality between CFC changes and increased large molecular permeability by histamine type mediators a 40% CFC increase would imply a decrease in effective transcapillary colloid osmotic pressure difference of at most ~ 3 mmHg (cf. Rippe, Kamiya & Folkow 1978). Thus most of the increased fluid filtration caused by 5-HT during the constant flow condition in this study is likely to be caused by capillary pressure elevations due to postcapillary vasoconstriction and only a small fraction due to pressure-independent mechanisms (cf. Kline et al. 1974).

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tively different exchange parameters can respond quite differently for given changes in either capillary permeability or surface area. Thus histamine induced permeability changes were generally well reflected by changes in capillary filtration coefficient (CFC) and capillary fluid equilibrium while capillary diffusion capacity for small molecules (PS) was very little affected. Changes in capillary surface area were on the other hand closely mirrored by PS changes while CFC always changed less.

Such discrepancies may be one of the main reasons for the inconsistencies between data from various laboratories concerning the effects of NA and 5-HT on capillary exchange. It would therefore be of interest to study simultaneously principally different exchange parameters in the same experiment to gain further insight into these problems. Towards such a background the present experiments were performed in which PS for Cr EDTA, CFC and capillary fluid equilibrium were simultaneously measured during well defined haemodynamic conditions and at constant flow during graded infusions of NA and 5-HT into initially maximally vasodilated rat hindquarters.

The NA induced relative changes in pre and postcapillary resistances (see Fig. 3) agree with recent measurements in rat by Folkow et al. 1977 though the absolute changes were moderately greater in the present study presumably because of higher flow rates. For 5-HT the pre and postcapillary resistance dose-response curves seemed to be fairly similar to those for NA though the postcapillary resistance increase seemed to be less marked for 5-HT. This was deduced from the filtration data in Fig. 2 and the CFC data in Fig. 5 under the assumption that 5-HT only induces minor P_c changes (up to 2–3 mmHg) during vasoconstriction which seems to be the case (see below).

NA infusion induced a dose related decrease in the effectively perfused capillary surface area as indicated by changes in both CFC and PS. Thus CFC-changes from 0.04 ml/min \times mmHg \times 100 g during maximal vasodilatation to resting values around 0.013–0.015 ml/min \times mmHg \times 100 g (cf. Cobbold et al. 1963) were associated with essentially proportional decreases in PS from 5.5–6 to about 1.5 ml/min \times 100 g (Fig. 4). However at higher NA doses PS for Cr EDTA was further reduced down to 0.6–0.8 ml/min \times 100 g while CFC decreased only to 0.011 ml/min \times mmHg \times 100 g. This type of discrepancy has frequently been observed earlier

though with the difference that the PS and CFC measurements were performed in different experiments (cf. Renkin 1969; Haddy, Scott & Goss 1976). Thus in the constant flow period of hindlimb (8–10 ml/min \times 100 g) Kaver & Dzau (1974) noted that CFC first decreased from rest values during the first two minutes of NA infusion and then generally returned to or slightly above control. In contrast Szwed & Tiedman (1975) during constant flow conditions (flow 1 ml/min \times 100 g) observed a marked and sustained decrease in ^{86}Rb extraction in dog gracilis muscle during vasoconstriction. Also when NA infusions or vasoconstrictor fibre stimulations cause flow reductions during constant pressure conditions it seems to be marked discrepancies between CFC and PS responses insofar as the former are said to increase slightly (Cobbold et al. 1963; Järhult 1971) while the latter seem to decrease considerably (Renkin & Rosell 1966; Appleton & Lewis 1968).

Concerning the background of these discrepancies between PS and CFC responses during induced vasoconstriction Szwed & Tiedman (1975) questioned the validity of CFC measurements as an indicator of capillary surface area compared with measurements of diffusion capacity. Renkin (1969) however suggested that the discrepancy rather reflects the complexity of the vascular network where increases in precapillary resistance may give different CFC/PS response patterns depending on which consecutive segments that are mainly constricted. Thus progressive constriction in an all-or none fashion of precapillary arterioles would give proportional decreases in CFC and PS while more graded constriction would change PS more than CFC since PS is flow dependent (Renkin 1969; Folkow & Mell 1970; Rippe, Kamiya & Folkow 1978).

The present data suggest that NA vasoconstriction from maximal vasodilatation to levels corresponding to in vivo resting resistance corresponds to a progressive increase coupled to a virtually complete constriction also of sphincter segment that reduces the number of perfused capillaries some 75%. However at further resistance increases the effect of CFC/PS changes suggests a predominant constriction of segment affecting overall resistance more than the microvascular flow distribution.

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Effects of purified macrophage RNases on granulation tissue fibroblasts with reference to silicosis

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AHO S, LEHTINEN J & KULONEN E. Effects of purified macrophage RNases on granulation tissue fibroblasts with reference to silicosis. *Acta Physiol Scand* 1980; 109: 275-281. Received 10 Nov 1979. ISSN 0001-6772. Department of Medical Chemistry, University of Turku, Finland.

Total alkaline RNases, designated RNase 1 and RNase 2, were isolated from the culture media of silica-treated and non-treated macrophages. The yield of RNase from the medium of silica-treated macrophages was 30% of that from the non-treated control. The effects of these RNases on cultured granulation tissue fibroblasts and on granulation-tissue nuclei were studied. RNase 1 inhibited thymidine incorporation into fibroblasts except at low concentrations, where it was observed to be stimulatory. RNase 1 also inhibited the protein synthesis of fibroblasts. The incorporation of cytidine into RNA in cultured fibroblasts was not affected by RNase 1 but the incorporation into isolated nuclei was decreased. In pulse chase experiments RNase 1 increased the release of cytidine, but not that of thymidine from the cells. RNase 2 had no effect on the protein or nucleic acid metabolism of the fibroblast or on the RNA metabolism of isolated nuclei, perhaps because of impermeability. These experiments confirm that macrophage RNase activity is able to regulate the metabolism of granulation-tissue fibroblasts by increasing RNA degradation. Through this action it also regulates DNA and protein synthesis and other metabolic functions of these cells.

Key words: RNA turnover, DNA synthesis, protein synthesis, macrophage RNases, fibroblasts, silica.

cells are stimulated to proliferate they first increase RNA (Mazuch & Green 1973; Johnson et al. 1976). The level of mRNA is a major rate-controlling factor in the synthesis of those proteins that are necessary for initiation and maintenance of DNA synthesis. Changes in RNA turnover cause a synthesis of proteins and finally also of DNA synthesis. Changes in RNA turnover cause a synthesis of proteins and finally also of DNA synthesis. Changes in RNA turnover cause a synthesis of proteins and finally also of DNA synthesis.

In this paper we have studied how the purified RNases from the media of cultured silica-treated and non-treated macrophages affect cultured granulation-tissue fibroblasts and nuclei isolated from experimental granulation tissue.

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MATERIALS AND METHODS

Culture of macrophages

Rat peritoneal macrophages were harvested and washed as described by Aho & Kulonen (1979). The washed cells were then transferred to culture flasks (Nunc, N1475) (10⁶ cells/174 cm² in 30 ml of serum-free Dulbecco's modification of Eagle's Minimum Essential Medium, DMEM, buffered with HEPES acid and NaHCO₃ and containing 100 IU/ml penicillin G and 90 µg/ml streptomycin sulphate) and allowed to adhere for 2 h. Nonadherent cells were removed by rinsing and fresh medium added. Where indicated the medium contained 0.25 mg/ml of SiO₂. Dörmann quartz DQ L, <5 µm. The cultures were incubated at 37°C in an atmosphere of 95% air plus 5% CO₂. After 2 and 4 days the medium was changed, both non-treated and treated cultures received medium without silica. The media were collected and filtered through Millipore-filter (0.22 µm) to remove detached cells and SiO₂-particles.

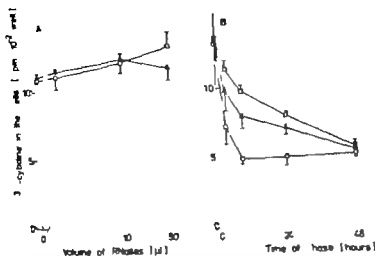


Fig. 2. The effect of RNase I on the incorporation (A) and release (B) of ^3H -cytidine in granulation-tissue fibroblasts. (A) Fresh culture medium with 2% FCS and RNase I is added to the confluent fibroblast cultures in microtest plates. After 4 h the cells are detached and collected, and the radioactivity determined. (B) Confluent cell cultures were labeled for 4 h with ^3H -cytidine in a medium containing 5% FCS. Medium with no serum and 90 μl of enzyme solution was added to the cells. At the indicated times the cells were detached and collected and the radioactivity determined. No enzyme added (O—O) RNase I from 3–4 day medium of non-treated (0.45 units/ml) (O—O) and of silica-treated (0.15 units/ml) (Δ—Δ) macrophages. Averages \pm S.E.M. (A: 4 B: 6) are presented.

Separation of radioactivity CTP into dATP

Incorporation of ^3H -CTP (TRK 339 The Radiochemical Centre, Amersham, U.K.), was measured by the method of Hooton & Hoffbrand (1977). The reaction mixture contained in total volume of 190 μl : 17 μl of dGTP, dCTP and dATP, 7 mM MgCl_2 , 0.1 M NaCl, 0.1 M ATP, 0.7 mM EDTA, 1 mM β -mercaptoethanol, 0.17 M Tris-HCl, pH 7.5, 0.2 μM ^3H -CTP corresponding to 75 μg DNA and 23 μl of the ^3H -RNases. The washed nuclei were hydrolyzed in 0.5 M KOH overnight at 37°C . The hydrolyzate was precipitated by adding 0.5 ml of 1.04 M perchloric acid and centrifuged at 1000 g for 15 min. The supernatant was decanted into a scintillation vial. The pellet was dissolved in 0.5 ml of 0.1 M perchloric acid, the acid combined with the first supernatant and the radioactivity in the samples determined as described by van (1979).

ULTS

Isolation of RNase from macrophage media

We separated two alkaline RNases from 60 ml medium of each silica-treated and non-treated macrophages. The volume of the final solutions from the poly(O)-Sepharose 4B

was adjusted to 6 ml. There were about equal amounts of RNase I and activities in the macrophage media (Fig. 1A). The 0–2 day medium of non-treated macrophages gave the highest RNase activity (0.90 units/ml = 100 used as reference value), the 3–4 day medium from non-treated cells and the 0–2 day medium from silica-treated cells gave lower RNase activity (0.50 and 0.45 units/ml respectively) and the 3–4 day medium from the silica-treated cells gave the lowest RNase activity (0.15 units/ml) (Fig. 1A).

The effects of macrophage RNase on granulation-tissue fibroblast

RNase 2 had no effect on the thymidine incorporation into cultured granulation-tissue fibroblasts, but the incorporation was inhibited by RNase 1 (Fig. 1B) depending on the amount of RNase activity in the medium. The low RNase 1 activity from the 3–4 day medium of silica-treated macrophages had no obvious effect but the higher RNase 1 activities from the three other media inhibited thymidine incorporation into granulation-tissue fibroblast by 50% (Fig. 1B).

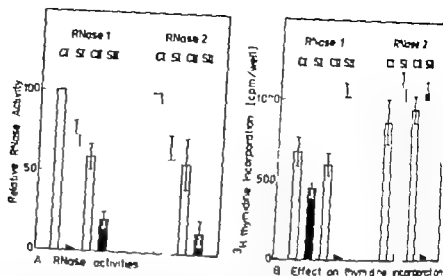


Fig. 1 The relative activities of RNases from macrophage media (A) and their effect on ³H thymidine incorporation into cultured granulation-tissue fibroblasts (B). RNases were purified from 60 ml of media from equal numbers of silica-treated and non-treated macrophages and the final volume of the RNase solutions was adjusted to 6 ml. The activities were determined with yeast RNA as substrate. ³H thymidine incorporation was determined in micro-titer plates in a total volume of 700 µl with 50 µl of RNase solution. RNase purified from the 0–3 day medium of non-treated (C I) and of silica-treated (S I) macrophages. C II RNase purified from the 3–4 day medium of non-treated and S II of silica-treated macrophages. Averages ± S.E.M. (n=4) are presented.

Purification of RNase from macrophage media

The RNases were isolated as described in detail elsewhere (Aho submitted for publication). The macrophage medium was first dialyzed against 0.05 M phosphate buffer pH 6 and then adsorbed onto an affinity column of protamine sulphate bound to Sepharose 4B (prepared according to Pharmacia instructions). The attached fraction was eluted with 1 M NaCl in phosphate buffer and desalted by dialysis against phosphate buffer. The attached desalted fraction and the non-attached fraction were separately chromatographed on an affinity column of poly(G) bound to Sepharose 4B and the material that attached were eluted with 1 M KCl and desalted by dialysis against phosphate buffer. The RNase attached to protamine sulphate was called RNase 1 and the non-attached one RNase 2.

Enzymic activity of the purified RNases was determined with yeast RNA as substrate as described by Aho & Kulonen (1979). One unit of enzyme produced 1 mmol of acid soluble nucleotides per hour in these conditions.

Production of experimental granulation tissue

Granulation tissue was induced in adult rat of Wistar strain by viscose cellulose sponge (Viljanto & Kulonen 1963). The granulation was collected after 14 days the surrounding capsules removed and the granulation used immediately.

Culture of granulation-tissue fibroblasts

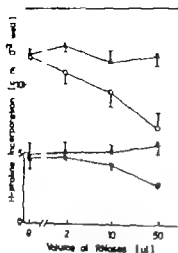
The sliced granulation was treated with collagenase trypsin and hyaluronidase and the cells isolated as described by Ivaska (1973). Cells were cultured in growth

medium as above but containing 10% fetal calf serum (FCS (Flow Laboratories)). When confluent, the cells were detached with 0.5% trypsin in Ca²⁺ Mg²⁺ free Ringer solution and then subcultured at a 1:4 dilution.

The incorporation of ³H-proline and ³H-cytidine was studied with confluent cells and that of ³H-thymidine with dividing cells at cell passages 4–8, using microtiter plates (Nunc/N1482/1-67008). The culture medium was changed and the sample added as indicated in Figures. After a 4 h incubation 0.1 µCi of [³H]-proline (TRK 333 The Radiochemical Centre, Amersham U.K.) (5-³H)-cytidine (TRK 195) or [³H]-thymidine (TRK 300) in 10 µl of medium was added at the incubation was continued for 24 h. The cells were detached with 0.5% trypsin, collected onto glass slides (OFIC Whatman) with an automatic cell harvester (made in our workshop) and the radioactivity in each determined as described by Runkama & Dobner (1977).

Isolation of nuclei from experimental granulation tissue

The granulation tissue was homogenized in TENM buffer (10 mM Tris HCl, 1 mM EDTA, 6 mM β-mercaptoethanol, 4 mM MgCl₂, pH 7.8) with an Ultra-Tam homogenizer (Janke & Kunkel, Staufen, Breisgau) using 1 g of tissue per 5 ml of buffer as described (Hershey et al. 1973). The homogenate was filtered through a nylon cloth and the nuclei recovered by centrifugation at 800g for 5 min. The pellet was washed three times with TENM buffer (10 mM Tris HCl, 1 mM EDTA, 6 mM β-mercaptoethanol, pH 8.0) as described by Hershey et al. (1973).



Effect of RNase I on ^3H -proline incorporation into proteins of cultured granulation-tissue fibroblasts. Fresh medium without FCS and with RNase I was added to confluent cell cultures. After 4 h incubation amount of incorporated ^3H -proline in the cells and secreted proteins is determined. The culture medium is collected on filter and the radioactivity determined. Effect of RNase I from 3-4 day incubation of non-treated (—○—○—) and of silica-treated (—●—●—) cells on the incorporation of ^3H -proline into cells (proteins) and into secreted proteins (closed symbols) are presented. S.E.M. ($n=4$) are presented.

silica-treated macrophages contains low RNase activity.

Effect of RNase I on the cell

ifferences in the molecular structures of RNase 1 and 2 are not yet clear. In SDS-acrylamide electrophoresis they have the same molecular weight (Aho submitted for publication). The present experiments proved that only RNase 1 had specific effects on granulation-tissue fibroblasts. The effects isolated from granulation tissue. The specific effects of ribonuclease preparations have been found to correlate well with the rate of uptake of ribonuclease (Bartholomew & Boudreau 1976). RNase A was taken up into cells 10 to 15 times faster than the monomeric RNase A. It is suggested that an RNA component is attached to macrophages (Aho submitted for publication). This role in the uptake of RNase into the cell is not cytotoxic concentrations (Fig. 3) the increased DNA synthesis could result from this RNA component. In prokaryotes there is a primer

RNA which must be cleaved off by a very specific RNase before the elongation and maturation of RNA-attached DNA segments to chromosomal DNA take place (Sheinin et al 1978).

Effect of RNases on cell metabolism

The activity of alkaline RNase controls the amount of polyribosomes and protein synthesis in various tissues (reviews of Levy 1974 and Dmoust & de Laminade 1975). The macrophage RNases rapidly degrade polysomal and isolated rRNA to smaller fragments (Aho & Kulonen 1980). There is no turnover of the 18S and 28S ribosomal RNA in growing cultures (Kolodny 1975) and a great portion of the ribosomes is in use. The degradation of rRNA in growing fibroblast cultures by macrophage RNase could greatly decrease the cellular metabolism. However in confluent cells the degradation of rRNA does not play the primary role in the regulation of protein synthesis (Waldron et al 1977) because slow-growing cells have a high proportion of inactive ribosomes. In this case mRNA is a more susceptible target for RNase. The mRNA in the cytoplasm and nucleus is normally protected from RNases by proteins (Spirm 1972, Barrieux et al 1976) and a poly(A)-tail (Marbais et al 1975, Heeter et al 1976) but it is susceptible to RNases during translation (Barnard 1969).

In this work the incorporation of cytidine and proline was studied in confluent cell cultures, which synthesize and secrete proteins but do not divide. In these cells the macrophage RNase decreased protein synthesis but the cellular metabolism was not decreased enough to be reflected as a decreased RNA synthesis during the 4 h incubation. The effect of RNase 1 on the nuclear RNA metabolism could be seen only after a 20 min incubation presumably because RNase 1 was not able to get through the nuclear membranes before. We suggest that RNase 2 could not get into the nuclei at all and, hence, had no effect on the cytidine incorporation.

The incorporation of thymidine was studied in rapidly growing granulation-tissue fibroblasts. In this case the effect of RNase 1 could be seen very clearly. With high amounts of RNase 1 the DNA synthesis was decreased as expected on the basis of earlier results. Low amounts of RNase increased thymidine incorporation into growing granulation-tissue fibroblasts. This may be due to the disturbance and destruction of some cellular components leading to reparative synthetic reactions, including

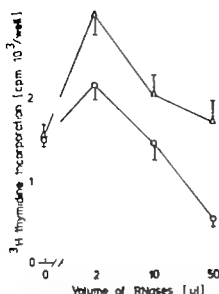


Fig. 3 Effect of RNase I on the incorporation of ^3H thymidine into cultured granulation-tissue fibroblast. Fresh culture medium with 5% FCS and RNase I was added to growing fibroblast cultures. After a 4 h incubation the amount of incorporated ^3H thymidine in the cell was determined. RNase I from 2–4 day medium of non-treated (O—O) and of silica-treated (Δ—Δ) macrophages. Averages \pm S.E.M. ($n=4$) are presented.

RNA synthesis of granulation-tissue fibroblasts was measured as ^3H -cytidine incorporation into confluent cultures, which no more synthesize DNA. RNase I had no effect on the incorporation (Fig. 2A). In pulse-chase experiments RNase I increased greatly the release of ^3H -cytidine from labeled RNA (Fig. 2B). It had no effect on the release of labeled thymidine from the granulation-tissue fibroblasts which were labeled during the growing phase (figures not presented).

The effect of RNase I on the incorporation of

^3H thymidine was dependent on its concentration. Small amounts of the enzyme (less than 4 el. 60 ng/well) stimulated the incorporation of thymidine, but greater amounts of the enzyme were inhibitory (Fig. 3). There was a linear correlation between the log of the enzyme activity and the incorporation of thymidine.

RNase I isolated from either the 0–2 day or 3–4 day medium of the silica-treated macrophages increased the incorporation of ^3H -cytidine into nuclear RNA about 50% when compared to the rest from an equal number of non-treated macrophages (Table 1). In contrast, no clear differences could be seen in the effects of RNase I from different media. The degradation of RNA could not be interpreted due to difficulties in separating rapidly CTP-labeled RNA from DNA. UTP does not incorporate in isolated nuclei of experimental granulocytes until destabilization of the nuclear membrane occurs.

RNase I inhibited ^3H -proline incorporation in granulation-tissue fibroblasts and into the secretory proteins. The inhibition was dependent on RNase activity (Fig. 4).

DISCUSSION

Effect of silica on the RNase activity

Silica crystals adsorb proteins on their surface from cell membranes causing membrane destruction (Kozm & McCarty 1976; Sumner et al. 1977). They also adsorb and inactivate RNases which are liberated from subcellular particles of peritoneal macrophages (Aho & Kulonen 1979). From cultured macrophages silica rapidly lyses and inactivates the RNases (Aho et al. 1979). As a consequence the medium from 3–4 day culture

Table 1 Effects of RNases on the incorporation of CTP into nuclear RNA in experimental granulation tissue

Nuclei were isolated from 14-day-old granulation tissue and incubated for 30 min with macrophage RNase in the presence of ^3H -CTP as described in Materials and Methods. Means \pm S.E.M. ($n=4$) are presented.

| Source of RNase | Incorporated radioactivity cpm/ μg DNA | |
|---|---|----------------|
| | RNase 1 | RNase 2 |
| Non-treated macrophage medium 0–2 days | 13.8 \pm 6.4 | 15.0 \pm 9.2 |
| Silica-treated macrophage medium 0–2 days | 21.5 \pm 9 | 17.6 \pm 7.9 |
| Non-treated macrophage medium 3–4 days | 14.9 \pm 8.8 | 20.8 \pm 7.1 |
| Silica-treated macrophage medium 3–4 days | 21.9 \pm 11.1 | 16.1 \pm 8.4 |

Effect of pretreatment with silica on RNase I from () 0–2 day medium $P < 0.05$ (b) 3

lum. $P < 0.05$

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DNA synthesis as discussed by Vasiliev et al (1970). In this work RNase I had no effect on the release of radioactivity from cells labeled with thymidine. However Lehtinen (1979) has shown that RNase I has some nuclease activity also on DNA of nuclei isolated from granulation tissue.

In conclusion the RNase activity in the macrophage culture medium varies with the age of the culture and is suppressed by silica treatment. The RNase which is liberated from macrophages disturbs the metabolism of fibroblasts and this effect depends on the molecular structure and concentration.

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Effects of chronic nerve conduction block on formation of neuromuscular junctions and junctional AChE in the rat

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CANGIANO A., LOMO T., LUTZEMBERGER L. & SVEEN O. Effects of chronic nerve conduction block on formation of neuromuscular junctions and junctional AChE in the rat. *Acta Physiol Scand* 1980; 109: 283-296. Received 12 Nov 1979. ISSN 0001-6772. Institute of Physiology, University of Oslo, Norway and Institute of Physiology, University of Pisa, Italy.

The development of ectopic n.m.j.s. between the transplanted superficial fibular nerve and the soleus muscle has been studied in adult rats. Impulse conduction in the sciatic nerve was blocked chronically and synapse formation between the blocked fibular nerve and the paralyzed soleus was compared with synapse formation between non-blocked fibular nerves and denervated soleus muscles. Nerves with conduction block readily made new n.m.j.s. Then 6 and 10-14 days after the onset of the block the number of newly innervated muscle fibres, the percentage of innervated fibres responding with action potentials and the frequency of m.p.p.s. at new junctions were comparable to that observed during innervation by non-blocked nerves. Muscle fibres innervated by both the original soleus nerve and the foreign fibular nerve were regularly encountered in the impulse blocked preparations. Junctions formed by impulse blocked fibular nerves had either no or very little AChE activity 10-15 days after the onset of the block. The evidence for this was 1) weak staining for ChE, 2) prolonged rise time and $\frac{1}{2}$ decay time of m.p.p.s., 3) positive correlation between m.p.p. amplitude and $\frac{1}{2}$ decay time and 4) insensitivity to anticholinesterases. In contrast, junctions formed by non-blocked fibular nerves had strong AChE activity by these criteria at corresponding times. AChE activity at the original soleus endplates was much reduced 10-15 days after the onset of conduction block.

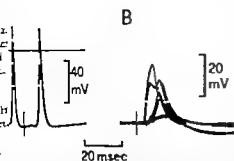
Key words: Neuromuscular junction formation, nerve conduction block, impulse activity, junctional AChE.

able of nerve impulse activity in the formation and maintenance of neuromuscular junctions has attracted considerable interest. Important questions underlying the stabilization (Stent & Changers & Dawson 1976), elimination (Kra et al 1977, Thompson et al. 1979) and extension (Gordon et al 1976, Renard et al. 1978) of nerve-muscle contact may all be influenced by nerve activity. On the other hand there is also evidence that many events of synapse formation occur in the absence of impulse activity (Harriss & Kudo 1976). Interest has also focused on junctional acetylcholinesterase (AChE) activity which present evi-

dence indicates is influenced both by evoked muscle activity (Giacomini et al 1973, Rubin et al 1978, Weinberg & Hall 1979, Lomo & Slater 1980b) and by neural substances (Fernandez & Inestrosa 1976) whose release is stimulated by impulse activity (Younkin et al 1978).

The aim of the present work is two-fold. First, to study synapse formation *in vivo* in the absence of impulse activity and second to see if in these circumstances, junctional AChE fails to appear as one would predict if impulse activity is of major importance.

We have used a preparation in which transplanted foreign nerve makes new ectopic synapses



B
Multifascicular innervation of single muscle fibres
days of nerve block. A, action potentials evoked by
stimulating the tibial nerve and then the fibular nerve
erect subthreshold endplate potentials evoked by
stimulating the fibular nerve with stimuli of increasing
intensity.

Tracor Northern NS-570 digital signal averager
the rising phase of each m.p.p. to trigger the

second group of expts. tape recorded on c.p.p.s
using a computer (NORD-1) which calculated the
mean rise time, decay time and amplitude of
each m.p.p. and mean values of these
parameters, usually based on 100 m.p.p.s from each
muscle. Amplitude histograms, frequency of m.p.p.
usually the Kendall's coefficient of rank correlation
between m.p.p. amplitude and decay time or rise time
or decay time of m.p.p. from normal soleus fibres
approximately twice as long in the first as in the
second group of expts. This difference was most probably
due to much lower room temperature, perhaps as
low as 10°C, in the first group of expts. Unfortunately
room temperature was not measured but must have
been much lower in the first group which was done in
the Physiology Laboratory in November-December while
the second group was done in very small laboratory
the room temperature usually lies around 25-30°C
and room temperature is known to increase the average
rise time of channel open times (Katz & Miledi 1972)
and decay time of m.p.p. (Boyd & Martin 1956).
For differences in absolute values, the two groups
gave similar results.

In summary, in nearly all expts, the distribution of
m.p.p.s is increased using the method of Buckley &
Miledi (1964). After physiological studies pairs of
tibia and fibula nerve block were then fixed
in aldehyde, dehydrated and cleared in benzyl benzoate.

RESULTS

Formation in the absence of nerve impulse activity

Application of a silicone rubber cuff around the
tibial nerve produced a clinically complete
paralysis in 19 animals. The paralysis developed
within about 1 day after the cuff had been applied

and lasted until the acute experiment 6-15 days
later.

The sciatic nerve and the soleus muscle were
then exposed and the nerve stimulated *in situ*. In all
19 animals stimulation below the cuff evoked strong
contractions in the soleus. The same stimulus
applied above the cuff evoked no microscopically
visible contraction in 14 animals (Fig. 1A) and weak
contractions in the remaining 5. In 8 animals with a
complete conduction block *in vivo* impulse conduc-
tion partially recovered when the cuff was removed
and the nerve muscle preparations placed in an
oxygenated Ringers solution since stimulation
above the cuff now caused weak contractions in the
soleus. The reasons for this recovery are not clear
but rapid return of nerve impulse conduction has
been described in humans after sudden relief from
chronic compression of the nerve (Hongell &
Mattson 1971).

In each of the soleus muscles with a successful
conduction block stimulation of the fibular nerve
below the cuff evoked c.p.p.s or action potentials in
surface fibres underlying the nerve growth (Table 1)
and a clear muscle contraction (Fig. 1C). The new
junctions were nearly always located several mm
proximal to the old endplates where the trans-
planted fibular nerve had grown into the soleus.
Stimulation of the tibial nerve evoked action po-
tentials in many but not all surface fibres (Table 1)
and a contraction which was usually much larger
than that evoked by stimulating the fibular nerve
(Fig. 1B). The tension produced by fibular nerve
stimulation was usually much greater when this
nerve stimulated alone (Fig. 1C) than when it was
superimposed on tibial nerve stimulation (Fig. 1D).
This indicates that most of the muscle fibres in-
nervated by the fibular nerve were also innervated
by the tibial nerve (Brown & Matthews 1960). That
the fibular nerve stimulation sometimes added some
tension to that produced by tibial nerve stimulation
alone (Fig. 1D) probably reflects the loss of tibial
nerve innervation on some muscle fibres through
damage from the cuff (Table 1). The presence of
double innervation was confirmed in many prepara-
tions by intracellular recordings from single muscle
fibres (Fig. 1A). Sometimes stimulation of the
fibular nerve evoked subthreshold c.p.p.s and in
such cases a gradual increase in stimulation in-
tensity frequently revealed several motoneuronal c.p.p.
(Fig. 1B). Evidently two or more fibular nerve
axons may innervate the same muscle fibre. Sub-

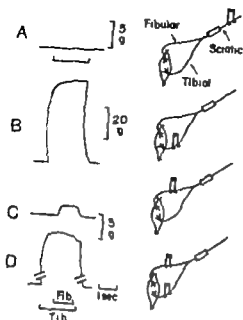


Fig. 1 Isometric responses of a doubly innervated soleus muscle to nerve stimulation after 9 days of nerve block by a compressive cuff around the sciatic nerve. Stimulation was *in vivo* at 100 Hz for 1 or 2 s. A stimulation of sciatic nerve above the cuff. B-D stimulation below the cuff of the tibial nerve alone in B, the fibular nerve alone in C and of the tibial and the fibular nerve together in D.

with a paralysed rat soleus muscle. The paralysis is caused by a cuff around the sciatic nerve which blocks impulse activity both in the original nerve and in the foreign nerve. This extends the work of Jansen et al. (1973) who used a similar preparation but did not deal with the effects of the cuff on the foreign nerve. By blocking the foreign nerve we could compare synapse formation by non-conducting and impulse-conducting nerves. With the notable exception that very little AChE developed in the blocked preparation there were no gross differences between the two types of preparation. In addition we have examined the effect of disuse on AChE activity at the original mature synapses and have extended and confirmed a previous report (Butler et al. 1978) of a considerable reduction in AChE activity.

METHODS

Preparation of animal. Male Wistar rat weighing 150–250 g were used. Under either of sodium pentobarbital anaesthesia the superficial fibular nerve was cut distally in both legs and moved to the dorsal surface of the proximal half of the soleus muscles (for further details see Lomo & Slater 1978). After ~1 week the animals were again anesthetized and an 8 mm long silicone rubber cuff (i.d.

1.0–1.1 mm o.d. 4 mm) placed around the nerve on one side. This caused complete paralysis of the leg within 1 day (see Cangiano et al. 1977 for further details). When this occurred the rats were anaesthetized at time and the contralateral soleus denervated by cutting the tibial nerve, leaving the transplanted fibular nerve intact. The animals were then examined clinically for up to 15 days. In most of the animals a complete paralysis of the lower leg persisted on the original final control was performed at the time of the experiment. The sciatic nerve was stimulated in 10–15 contractions in the soleus muscle looked for activity at the dissection microscope. In most rats proximal stimulation produced no visible response while distal stimulation elicited a vigorous muscle contraction. These animals were retained for further study. Evidently in these rats nerve conduction had been successfully and powerfully blocked at the site of the cuff. The remaining animals had incomplete conduction blocks at the time of the experiments usually also had shown clinical improvement from paralysis.

Contractile responses of the soleus to nerve stimulation proximally and distally to the cuff were also made. Isometric tension recordings (Fig. 1). This was done *in vivo* by connecting the Achilles tendon to a wire (after the distal bellies of the medial and lateral peroneus muscles had been resected).

Acute experiment. The soleus muscles, sciatic nerve and the foreign nerve attached were removed from legs 6 to 15 days after onset of paralysis by cuff block or section of the tibial nerve. The muscles were perfused in a chamber at room temperature with a maltan saline solution equilibrated with 95% O₂ and CO₂ (pH 7.3). The Ringer contained (mM): Na⁺ 119, K⁺ 4, Ca²⁺ 1, Mg²⁺ 1, H₂PO₄⁻ 1, HCO₃⁻ 12, Cl⁻ 145, glucose 10.

Miniature e.p.p.s. evoked e.p.p.s. and action potentials were recorded intracellularly with conventional microelectrodes filled with 4 M K-acetate or 3 M KCl in both (fibular nerve) and old (soleus nerve) junctions. Old plates were localized by following the terminal fibres of the soleus nerve in the microscope and direct recording sites where the m.e.p.p.s. are large and rising. The new fibular synapses on the other hand lacked a precise morphological landmark and the recording site was not necessarily very close to the junctional area. Furthermore many muscle fibres had multiple and distributed synaptic contacts (Lomo & Slater 1978). In any given fibre several penetrations (150–400 µm) were therefore made and records taken from sites of a substantial fraction of the m.e.p.p.s. were large and rising.

In many experiments Neostigmine methiodide (Rixhe) was added to the bathing fluid to reverse a concentration of 10⁻⁶ g/ml. In other experiments the junctional ACh sensitivity was measured by iontophoresis of ACh and the sensitivity expressed as depolarization per nanocoulomb charge ejected from AChCl (3M) containing pipette.

Computations of m.e.p.p. amplitude and time course. In an initial group of experiments the decay time and amplitude of usually 100 m.e.p.p.s. at each junction were obtained by averaging the period

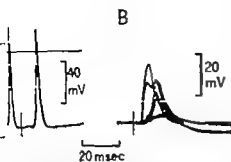


Figure B. Intracellular recordings of single muscle fibres after nerve block. A, action potentials evoked by stimulating the tibial nerve and then the fibular nerve. B, subthreshold endplate potentials evoked by stimulating the fibular nerve with stimulus of increasing intensity.

Trace Northern NS-570 digital signal averager. The rising phase of each e.p.p. to trigger the

second group of experiments recorded in e.p.p. also computer (NORD-1) which calculated the average rise time, decay time and amplitude of e.p.p.s. The mean and median values of these parameters, usually based on 100 e.p.p.s from each animal, are plotted in histograms. Frequency of e.p.p.s is calculated by the Kendall's coefficient of rank correlation between e.p.p. amplitude and decay time or rise time. Decay time of e.p.p.s from normal soleus fibres is approximately twice as long as the first as in the first group of experiments. This difference was most probably due to much lower room temperature, perhaps as low as 10°C in the first group of experiments. Unfortunately room temperature was not monitored but must have been much lower in the first group which was done in a laboratory in November-December. A whole second group as done in very small laboratory at the room temperature usually lies around 25-30°C. The temperature is known to increase the average time course of e.p.p.s (Katz & Miledi 1977) and decay time of e.p.p.s (Boyd & Martin 1956). For differences in absolute values, the two groups are quite similar results.

Microscopy. In nearly all experiments, the distribution of innervation was determined using the method of Buckley & Martin (1966). After physiological studies pairs of nerves and tibial nerve block were then fixed in alcohols, dehydrated and cleared in benzyl benzoate.

RESULTS

Formation in the absence of nerve block

In 19 animals, a silicone rubber cuff around the tibial nerve produced a clinically complete paralysis about 1 day after the cuff had been applied

and lasted until the acute experiment 6-15 days later.

The sciatic nerve and the soleus muscle were then exposed and the nerve stimulated *in situ*. In all 19 animals stimulation below the cuff evoked strong contractions in the soleus. The same stimulus applied above the cuff evoked no macroscopically visible contraction in 14 animals (Fig. 1A) and weak contractions in the remaining 5. In 8 animals with a complete conduction block *in vivo* impulse conduction partially recovered when the cuff was removed and the nerve muscle preparations placed in an oxygenated Ringers solution since stimulation above the cuff now caused weak contractions in the soleus. The reasons for this recovery are not clear but rapid return of nerve impulse conduction has been described in humans after sudden relief from chronic compression of the nerve (Hongell & Mattson 1971).

In each of the soleus muscles with a successful conduction block, stimulation of the fibular nerve below the cuff evoked e.p.p.s or action potentials in surface fibres underlying the nerve growth (Table 1) and a clear muscle contraction (Fig. 1C). The new junctions were nearly always located several mm proximal to the old endplates where the transplanted fibular nerve had grown into the soleus. Stimulation of the tibial nerve evoked action potentials in many but not all surface fibres (Table 1) and contraction which was usually much larger than that evoked by stimulating the fibular nerve (Fig. 1B). The tension produced by fibular nerve stimulation was usually much greater when this nerve stimulated alone (Fig. 1C) than when it was superimposed on tibial nerve stimulation (Fig. 1D). This indicates that most of the muscle fibres innervated by the fibular nerve were also innervated by the tibial nerve (Brown & Matthews 1960). That the fibular nerve stimulation sometimes added some tension to that produced by tibial nerve stimulation alone (Fig. 1D) probably reflects the loss of tibial nerve innervation on some muscle fibres through damage from the cuff (Table 1). The presence of double innervation was confirmed in many preparations by intracellular recordings from single muscle fibres (Fig. 1A). Sometimes stimulation of the fibular nerve evoked subthreshold e.p.p.s and in such cases a gradual increase in stimulation intensity frequently revealed several unitary e.p.p.s (Fig. 2B). Evidently two or more fibular nerve axons may innervate the same muscle fibre. Sub-

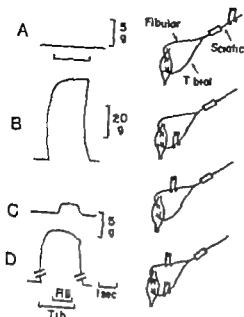


Fig. 1. Isometric responses of a doubly innervated soleus muscle to nerve stimulation after 9 days of nerve block by a compressive cuff around the sciatic nerve. Stimulation was in vivo at 100 Hz for 1 or 2 s. A: stimulation of sciatic nerve above the cuff; B-D: stimulation below the cuff of the tibial nerve alone in B, the fibular nerve alone in C and of the tibial and the fibular nerve together in D.

with a paralysed rat soleus muscle. The paralysis is caused by a cuff around the sciatic nerve which blocks impulse activity both in the original nerve and in the foreign nerve. This extends the work of Jansen et al. (1973) who used a similar preparation but did not deal with the effects of the cuff on the foreign nerve. By blocking the foreign nerve we could compare synapse formation by non-conducting and impulse-conducting nerves. With the notable exception that very little AChE developed in the blocked preparation there were no gross differences between the two types of preparation. In addition we have examined the effect of disuse on AChE activity at the original mature synapses and have extended and confirmed a previous report (Butler et al. 1978) of a considerable reduction in AChF activity.

METHODS

Preparation of animal. Male Wistar rat weighing 150–250 g were used. Under ether or sodium pentobarbital anaesthesia the superficial fibular nerve was cut distally in both legs and moved to the dorsal surface of the proximal half of the soleus muscles (for further detail see Lomo & Slater 1978). After 2–3 weeks the animals were again anesthetized and an 8 mm long silicone rubber cuff (I.D.

1.0–1.1 mm i.d. 4 mm) placed around the nerve on one side. This caused complete paralysis of the within 1 day (see Cangiano et al. 1977 for further details). When this occurred the rats were anesthetized at time and the contralateral soleus denervated in or the tibial nerve, leaving the transplanted fibular nerve intact. The animals were then examined clinically for up to 15 days. In most of the animals a complete paralysis of the lower leg persisted on the initial clinical control was performed at the time of the experiment. The sciatic nerve was stimulated and contractions in the soleus muscle looked for under the dissection microscope. In most rats proximal stimulation produced no visible response while distal stimulation elicited a vigorous muscle contraction. These animals retained for further study. Evidently in these rats, conduction had been successfully and persistently blocked at the site of the cuff. The remaining rats had incomplete conduction blocks at the time of the experiments usually also had shown clinical signs of recovery from paralysis.

Contractile responses of the soleus to nerve stimulation proximally and distally in the cuff were also studied. Isometric tension recordings (Fig. 1). This was done in vivo by connecting the Achilles tendon to a strain gauge after the distal bellies of the medial and lateral plantaris muscles had been resected.

Acute experiment. The soleus muscles of the legs 6 to 15 days after onset of paralysis by cuff block or section of the tibial nerve. The animals were perfused in a chamber at room temperature with a mullikan saline solution equilibrated with 95% O₂ and 5% CO₂ (pH 7.3). The Ringer contained (mM): Na 140, K 5, Ca 2, Mg 1, H₂PO₄ 1, HCO₃ 12, Cl 124, glucose 10.

Miniature e.p.p.s. evoked e.p.p.s. and action potentials were recorded intracellularly with concentric recording pipettes filled with 4 M K-acetate or 3 M CsCl at both old (fibular nerve) and old (soleus nerve) junctions. Old plates were localized by following the terminal of the soleus nerve in the microscope and closed recording sites where the m.e.p.p.s. were large and frequent. The new fibular synapses on the other hand lacked a precise morphological landmark and the recording site was not necessarily very close to the distal area. Furthermore many muscle fibres had multiple and distributed synaptic contacts (Lomo & Slater 1978). In any given fibre several penetrations (250–500 µm) were therefore made and records taken from several. A substantial fraction of the m.e.p.p.s. were large and frequent.

In many experiments Neostigmine methiodide (Roche) was added to the bathing fluid to obtain a concentration of 10⁻⁶ g/ml. In other experiments the junctional ACh sensitivity was measured by microphoresis of ACh and the sensitivity is expressed as depolarization per nanocoulomb charge ejected from AChCl (3M) containing pipette.

Computation of m.e.p.p. amplitude rise time and decay time. In an initial group of experiments the rise time and amplitude of a highly 100 mV m.e.p.p. each junction were 0.5 a criterion for

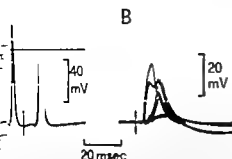


Figure B. Multichannel oscilloscope traces of single muscle fibres days of nerve block. A, action potentials evoked by stimulating the tibial nerve and then the fibular nerve. B, subthreshold endplate potentials evoked by stimulating the fibular nerve with stimuli of increasing intensity.

Tracor Northern NS-570 digital signal analyzer the rising phase of each e.p.p. to trigger the

second group of experiments recorded m.e.p.p.s and computer (HORD-1) which calculated the following values: rise time, $\frac{1}{2}$ decay time and amplitude of each e.p.p., mean and median values of these parameters, usually based on 100 m.e.p.p.s from each animal, amplitude histograms, frequency of rise e.p.p.s and the Kendall coefficient of rank correlation between m.e.p.p. amplitude and $\frac{1}{2}$ decay time or rise time. $\frac{1}{2}$ decay time of m.e.p.p.s from normal soleus fibres is approximately twice as long as the first as in the first group of experiments. This difference was most probably caused by such lower room temperature, perhaps as low as 18°C, in the first group of experiments. Unfortunately room temperature was not measured but must have been much lower as the first group which was done in the laboratory in November–December while the second group as done in very small laboratory in the room temperature usually lies around 25–30°C. The temperature is known to increase the average time course of channel open time (Katz & Miled 1972) and $\frac{1}{2}$ decay time of m.e.p.p.s (Boyd & Martin 1956) but for differences in absolute values, the two groups give similar results.

The histogram is nearly all experiments, the distribution of m.e.p.p.s is determined using the method of Buckley & Martin (1966). After physiological studies pairs of experiments with and without nerve blocks were then fixed, dehydrated and cleared in benzyl benzoate.

RESULTS

Neuromuscular junction formation in the absence of nerve impulse activity

Application of a silicone rubber cuff around the sciatic nerve produced classically complete paralysis in 19 animals. The paralysis developed within about 1 day after the cuff had been applied

and lasted until the acute experiment 6–15 days later.

The sciatic nerve and the soleus muscle were then exposed and the nerve stimulated *in situ*. In all 19 animals stimulation below the cuff evoked strong contractions in the soleus. The same stimulus applied above the cuff evoked no microscopically visible contraction in 14 animals (Fig. 1A) and weak contractions in the remaining 5. In 8 animals with a complete conduction block *in vivo* impulse conduction partially recovered when the cuff was removed and the nerve muscle preparations placed in an oxygenated Ringers solution since stimulation above the cuff now caused weak contractions in the soleus. The reasons for this recovery are not clear but rapid return of nerve impulse conduction has been described in humans after sudden relief from chronic compression of the nerve (Hoogell & Mattson 1971).

In each of the soleus muscles with a successful conduction block stimulation of the fibular nerve below the cuff evoked e.p.p.s or action potentials in surface fibres underlying the nerve growth (Table 1) and a clear muscle contraction (Fig. 1C). The new junctions were nearly always located several mm proximal to the old endplates where the transplanted fibular nerve had grown into the soleus. Stimulation of the tibial nerve evoked action potentials in many but not all surface fibres (Table 1) and a contraction which was usually much larger than that evoked by stimulating the fibular nerve (Fig. 1B). The tension produced by fibular nerve stimulation was usually much greater when this nerve stimulated alone (Fig. 1C) than when it was superimposed on tibial nerve stimulation (Fig. 1D). This indicates that most of the muscle fibres innervated by the fibular nerve were also innervated by the tibial nerve (Brown & Matthews 1960). That the fibular nerve stimulation sometimes added some tension to that produced by tibial nerve stimulation alone (Fig. 1D) probably reflects the loss of tibial nerve innervation on some muscle fibres through damage from the cuff (Table 1). The presence of double innervation was confirmed in many preparations by intracellular recordings from single muscle fibres (Fig. A). Sometimes stimulation of the fibular nerve evoked subthreshold e.p.p.s and in such cases a gradual increase in stimulation intensity frequently revealed several unitary e.p.p.s (Fig. 2B). Evidently two or more fibular nerve axons may innervate the same muscle fibre. Sub-

Table 1 Surface fibres responding with e.p.p.s or action potentials to stimulation of fibular nerve. During fibular nerve stimulation only fibres underneath the growth of the fibular nerve were examined

| | Days of paralysis | Innervated fibres (%) | Innervated fibres with AP (%) | Number of fibres | Number of muscles |
|---------------------------|-------------------|-----------------------|-------------------------------|------------------|-------------------|
| Fibular nerve not blocked | 6 | 77 | 37 | 25 | 1 |
| | 10-14 | 89 | 69 | 89 | 1 |
| Fibular nerve blocked | 6 | 88 | 57 | 3 | 1 |
| | 10-14 | 87 | 72 | 149 | 5 |
| Soleus nerve blocked | 6 | 77 | 77 | 5 | 1 |
| | 10-11 | 54 | 54 | 56 | 1 |

threshold multiple e.p.p.s were particularly frequent in the early stages of reinnervation. The findings confirm those reported by Jansen et al (1973) and extend them by showing that the fibular nerve forms synapses even when the conduction block affects the fibular nerve itself.

Many of the findings in the nerve impulse blocked soleus muscles were indistinguishable from those obtained when the soleus muscle was denervated and the transplanted fibular nerve left intact and allowed to conduct impulses. In both types of preparation the extent of the foreign nerve growth was roughly similar and muscle fibres innervated by the fibular nerve were found with similar ease in both. Thus after 6 and 10-14 days the blocked fibular nerve had innervated 88% and 87% respectively of the surface fibres lying underneath the nerve growth while the corresponding figures for the unblocked fibular nerve were 77% and 89% (Table 1).

There was no indication that the impulse blocked fibular nerve made unstable or transient synapses. In muscles innervated by the intact fibular nerve the mean frequency of spontaneous m.e.p.p.s more

than doubled between the 6th and the 10-14 (Table 2). During this period the endplates became more mature as shown by an increase in the number of evoked e.p.p.s that reaches threshold for discrete action potentials (Lomo & Slater 1975), by the appearance of junctional AChE (Lomo & Slater 1975) and below) and by the extent of their maturation (Kornelissen & Sommerschildt 1975). Transmission at synapses made by the blocked nerve was not obviously different from transmission at synapses made by the intact nerve. At 10-14 days both the frequency of spontaneous m.e.p.p.s (Table 2) and the effectiveness of transmission as judged by the percentage of innervated fibres responding with action potentials to stimulation of the nerve were roughly similar in the two types of preparation (Table 1). The relatively small number of fibres in both preparations with sub-threshold e.p.p.s may be attributed to several factors such as 1) immature synapses releasing only small amounts of transmitter, 2) low resting membrane potentials resulting from the foreign paralysis and 3) effects of the cuff or the anaes-

Table 2 Characteristics of m.e.p.p.s including Kendall's coefficient of correlation (τ) between amplitude and rise time at ectopic junctions formed by fibular nerve

§ E. of the mean indicated

| m.e.p.p. characteristics | | | | | | | |
|---------------------------|-------------------|-----------------|------------------|------------------|-------------------------|------------------|-------------------|
| | Days of paralysis | No./sec | Rise time (msec) | Amplitude (mV) | amplitude vs. rise time | Number of fibres | Number of muscles |
| Fibular nerve not blocked | 6 | 0.41 ± 0.1 | 3.03 ± 0.21 | 0.39 ± 0.037 | 0.1 ± 0.04 | 8 | 2 |
| | 10-14 | 1.02 ± 0.14 | 1.14 ± 0.03 | 0.36 ± 0.015 | 0.02 ± 0.01 | 22 | 2 |
| Fibular nerve blocked | 10-14 | 1.22 ± 0.70 | 1.80 ± 0.05 | 0.44 ± 0.024 | 0.06 ± 0.03 | 23 | 2 |

3 Surface fibres responding with 1 or more clearly distinguishable e.p.p.s to stimulation of fibular nerve at increased intensity

| | Days of paralysis | Fibres innervated by 1, 2, 3 or 4 axons (%) | | | | Number of fibres | Number of muscles |
|-------------------|-------------------|---|----|----|---|------------------|-------------------|
| | | 1 | 2 | 3 | 4 | | |
| nerve not blocked | 14 | 79 | 17 | 4 | | 24 | 2 |
| nerve blocked | 6 | 14 | 57 | 28 | | 7 | 1 |
| | 10-14 | 58 | 30 | 10 | 2 | 50 | 3 |

action block on transmitter release. That the factors may be important was indicated by the observation that after 10-14 days of conduction block many e.p.p.s evoked by stimulation of the nerve failed to elicit action potentials (Table 1). Thompson et al. (1979) have observed a similar phenomenon in newborn rats where conduction block of the sciatic nerve had been chronically induced by plugs containing TTX which did not cause nerve degeneration. In these circumstances occurred striking reduction in indirect tension attributable to generation of sub-threshold e.p.p.s.

Neuronal innervation of single muscle fibres is a characteristic feature of ectopic synapse formation in this preparation (Kuffler et al. 1977; Lomo & Slater 1980a). Also in this respect synapses made by blocked and unblocked fibular nerves were similar. As shown in Table 3 there was no qualitative difference between the two types of nerve fibres in capacity to multiply innervate single muscle fibres. There is suggestion of more extensive collateral innervation in the blocked preparation but more data are required to establish if this is true.

Action of AChE in new synapses

The present study indicated striking differences in junctional AChE activity at synapses made by impulse blocked and non-blocked fibular nerves. AChE activity at neuromuscular junctions can be demonstrated both histochemically and by the activity of endplate potentials to AChE inhibitors. These inhibitors increase the amplitude and prolong the time course of m.e.p.p.s probably by allowing released ACh to diffuse in the synaptic cleft and react with ACh receptors (Katz & Lebel 1973). This also results in a positive correlation between amplitude and $\frac{1}{2}$ decay time of e.p.p.s not normally present (Fig. 10A). We have

used these different indicators of junctional AChE activity to study its development at synapses made by either intact or impulse blocked fibular nerves.

Histochemistry. The ChE staining characteristics of denervated and nerve impulse blocked muscles were markedly different. 8 or more days after section of the tibial nerve numerous intense and often well circumscribed plaques were present underneath the growth of impulse conducting fibular nerves (Pl. 1A) confirming previous results (Lomo & Slater 1980b). In contrast, there were only faint, elongated streaks of deposits in nerve impulse blocked muscles (pl. 1C). The faint deposits in Pl. 1C were typical of 9 nerve impulse blocked muscles all of which contracted on stimulation of the fibular nerve. The deposits were present only in the region where the fibular nerve had made new synapses. Their significance is uncertain since different kinds of ChE can be found along growing nerve terminals not making junctional synapses (Doeben 1970).

Time course of m.e.p.p.s. 6 days after the onset of paralysis by denervation or nerve conduction block the duration of m.e.p.p.s was longer than normal in both types of preparation. Between 6 and 10 days the $\frac{1}{2}$ decay time and the rise time decreased markedly in muscles innervated by impulse conducting fibular nerves (Fig. 3 Table 2). This decrease coincides in time with the histochemical appearance of AChE activity (Lomo & Slater 1980b). In the nerve impulse blocked muscles on the other hand, the reduction in m.e.p.p. duration was much less pronounced. After 10-14 days the $\frac{1}{2}$ decay time was 71% (Fig. 4A) and the rise time 58% (Table 2) longer than in the corresponding impulse conducting preparation. The reduction which did occur may be attributed to (1) appearance of small amounts of AChE, (2) inactivation of adjacent synapses (Kuffler et al. 1977; Lomo & Slater 1980) reducing the contribution of m.e.p.p.s with a long time course due to electrotonic attenuation and (3) pos-

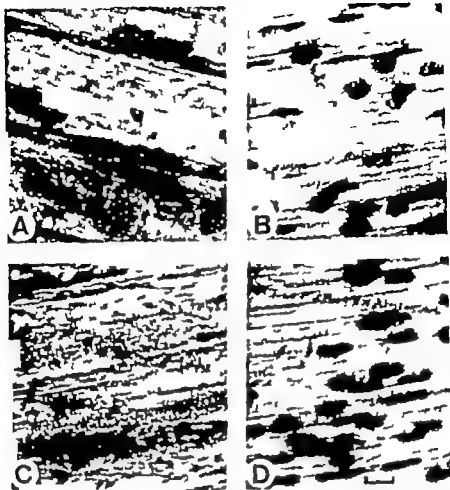


Plate 1 Representative deposits after staining for ChE (Buckley and Heaton 1968) in a soleus muscle denervated for 10 days (A and B) or paralysed for 10 days by a sciatic nerve block (C and D). A and C are from ectopic junctions formed by non-blocked and blocked transplanted fibular nerves respectively. B and D from denervated and nerve blocked original soleus endplates respectively. The two muscles are from the same rat and were stained together under identical conditions.

sible changes in the kinetics of the processes underlying transmission during synaptic maturation (Sakmann & Brenner 1978).

MEPP amplitude vs. $\frac{1}{2}$ decay time. An increase in $\frac{1}{2}$ decay time is no unequivocal sign of reduced AChE activity. A similar increase would be caused by an increased specific membrane resistance (R_m) or capacitance (C_m), whereas changes in fibre diameter or internal resistance would have no such effect (see Gage 1976). Denervation causes R_m to increase (Westgaard 1975) and this may occur also after a chronic nerve impulse block which resembles denervation in many of its other effects (Lomo & Rosenthal 1977; Cangiano et al. 1977). An increased R_m however would not be expected to affect the correlation between MEPP amplitude and $\frac{1}{2}$ decay time (or rise time). Normally there is no

correlation between these parameters (Fig. 10A) but if AChE is inhibited a positive correlation appears (Fig. 10B). We have therefore examined the correlation in impulse blocked muscles to obtain evidence for a reduced AChE activity which is independent of changes in R_m .

Fig. 5 (upper part) shows scatter diagrams of MEPP amplitude against $\frac{1}{2}$ decay time for representative fibres. Fig. 5A is from a 6-day denervated muscle and the observed positive correlation ($r=0.51$) is consistent with lack of AChE activity. Common at this time is also the skew amplitude distribution and the low frequency of MEPPs (here 0.17 Hz). At 10 days a positive correlation is no longer present in a denervated fibre ($r=0.04$) but still present in a nerve impulse blocked fibre ($r=0.54$). Fig. 4B and C show the data treated

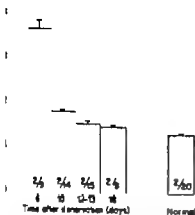


Fig. 3. Reduction in $\frac{1}{2}$ decay time of m.e.p.p.s at new motor nerve junctions with increasing time after the sciatic nerve. Bars give s.e. of the mean. Coll. refers to normal sciatic junctions. Number of and fibres examined is indicated.

After 10–14 days there is on average a positive correlation in nerve impulse $\frac{1}{2}$ fibres ($r=0.37$, $P<0.02$) compared with ated fibres ($r=0.14$, $P>0.35$). The correlation have been larger had all the m.e.p.p.s mented at one site. Instead some fibres had more separated inputs causing small, attenu e.p.p.s to be recorded together with larger ones. This tends to give a negative correlation.

of Neostigmine. If it is true that the new es in nerve impulse blocked muscles fail to junctional AChE, then eserine inhibitors i Neostigmine should have little or no effect p.p.s generated at these synapses. In denerv muscles, on the other hand the m.e.p.p.s be prolonged. Fig. 6A, B shows that these axons are fulfilled. 13–15 days after the onset of the $\frac{1}{2}$ decay time is relatively long in the impulse blocked muscles (Fig. 6B) and on e only slightly prolonged by the addition of of Neostigmine to the perfusion fluid. The pation was not statistically significant at the vel. In denervated muscles, on the other the $\frac{1}{2}$ decay time was initially relatively short cocaine prolonged it markedly (Fig. 6A). of these results were obtained by recording the same identified fibres before and after ation of Neostigmine. The experiments with apine are done at a relatively low temperature Methods), and this probably accounts for

relatively long duration of m.e.p.p.s in these experiments. Such an effect of temperature has been described previously (Boyd & Martin 1956).

Preparations with incomplete nerve impulse block. In a few rats the clinical paralysis began to wear off 10–15 days after the cuff had been applied. At this time the soleus muscle contracted weakly to stimulation of the sciatic nerve above the cuff indicating that some axons had recovered from the conduction block. These preparations were interesting because they offered an opportunity to study the time course of m.e.p.p.s in muscle fibres with varying degrees of previous activity. The level or duration of this activity is not known but an indication may be obtained by measuring the extrajunctional ACh sensitivity which is known to depend critically on the amount or type of previous muscle activity (Lomo & Westgaard 1975). Fig. 7 gives the result from such an incompletely blocked muscle. It is seen that the muscle fibres with the higher extrajunctional ACh sensitivity also had m.e.p.p.s with the longer rise times and $\frac{1}{2}$ decay times. Furthermore it was observed that fibres with high extrajunctional ACh sensitivity were thin and stained weakly for ChE at the end of the experiment while fibres with low extrajunctional ACh sensitivity were thick and stained intensely for ChE where the m.e.p.p.s had been recorded. If the longer m.e.p.p. rise times and $\frac{1}{2}$ decay times reflect reduced junctional AChE activity then the correlation shown in fig. 7 indicates that induction of junctional AChE and removal of extrajunctional ACh sensitivity are correlated phenomena, both

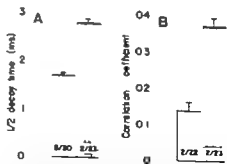


Fig. 4. $\frac{1}{2}$ decay time of m.e.p.p. longer (A) and Kendall's correlation coefficient between $\frac{1}{2}$ decay time and amplitude of m.e.p.p. more positive (B) at junctions formed by blocked fibular nerves (hatched columns) than by non-blocked fibular nerves (open columns) 10–14 days after onset of muscle paralysis. Bars give s.e. of the mean. Number of muscles and fibres examined is indicated.

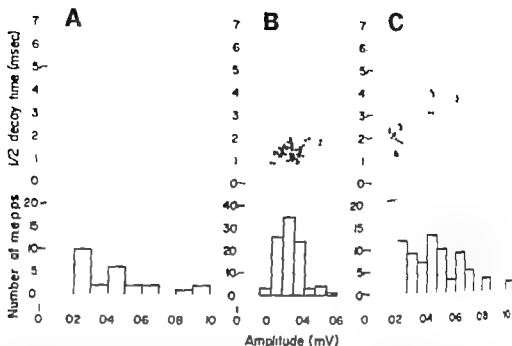


Fig. 5 Scatter diagram of $\frac{1}{2}$ decay times vs. amplitude of individual m.e.p.p.s from a non-blocked fibular nerve junction (A) and 10 (B) days after cutting the soleus nerve and from a blocked fibular nerve junction 10 days after onset of paralysis (C) (upper row). Lower row shows distribution of m.e.p.p. amplitudes from the same 3 junctions.

probably depending on muscle activity in a graded manner.

Junctional AChF at original nerve impulse blocked synapses. In these preparations impulse conduction was blocked not only in the foreign nerve but also in the original nerve to the soleus muscle. It was therefore possible to examine how lack of impulse activity affected the maintenance of AChE at well established mature synapses.

Histochemistry. Histochemical staining for ChE did not give any clear indication of an effect of the nerve block. Both nerve impulse blocked and innervated original synapses stained well for ChE (Fig. 1B-D) and neither differed notably from the staining of normal synapses. We therefore looked for more subtle changes by examining the m.e.p.p.s at the nerve impulse blocked synapses along the lines described above for the new synapses.

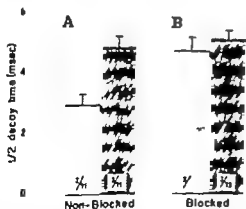


Fig. 6 Effect of Neostigmine on $\frac{1}{2}$ decay time of m.e.p.p.s at ectopic junctions formed by non-blocked (A) and blocked (B) fibular nerves 13-15 days after onset of muscle paralysis. Open columns before and hatched columns after addition of 10^{-6} g/ml of Neostigmine to the perfusion fluid. Bars give S.E. of the mean. Number of muscles and of fibres is indicated.

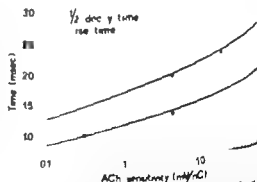


Fig. 7 Positive correlation between m.e.p.p. $\frac{1}{2}$ decay time (●) or rise time (○) and extrajunctional ACh sensitivity. The fibres from which the m.e.p.p.s were obtained had already recovered from the block starting 17 days earlier. Kendall's correlation coefficients were 0.74 ($\frac{1}{2}$ decay time) and 0.83 (rise time).

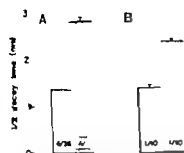


Fig. 8. 1/2 decay times of m.e.p.p.s. at original soleus endplates in normal muscles (A) and in open columns), after 4 days of conduction block in the tibial nerve (A, hatched columns) and after addition of 10^{-5} g/ml Neostigmine to the perfusion fluid (B, hatched columns). Bars 1 S.E. of the mean. Number of muscles and of fibres is stated.

effects of m.e.p.p.s. The chronic nerve conduction block caused a significant increase in the duration of the m.e.p.p.s. at the original endplates. On average the rise time became approximately longer (Table 4) and the 1/2 decay time approximately 100% (Fig. 8A) longer than normal after 4 days of conduction block. Similar but less marked increases occurred at normal soleus endplates after Neostigmine (10^{-5} g/ml) had been added to the perfusion fluid (Fig. 8B).

m.e.p.p. amplitude vs. 1/2 decay times Chronic conduction block of 10–14 days duration induced the appearance of a positive correlation between m.e.p.p. 1/2 decay time and amplitude. The Kendall coefficient of correlation (τ) was 0.28 and correlation was significantly positive at the 1% level for 90% of the fibres (Fig. 9C). In normal muscles, on the other hand, the median was only 0.08 (Fig. 9A) and only 6% of the fibres showed significant correlation at the 1% level. The positive correlation induced by the block may be compared

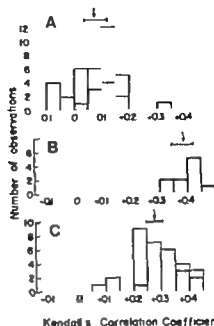


Fig. 9. Distribution of Kendall's coefficient of correlation between 1/2 decay time and amplitude of m.e.p.p.s. at original soleus endplates in normal muscles (A), in normal muscles poisoned to 10^{-5} g/ml of Neostigmine (B) and in muscles paralysed for 10–12 days by chronic sciatic nerve block (C). Hatched columns in A and B represent subpopulation of normal fibres before (A) and after (B) application of Neostigmine and in C fibres from the longest paralysed muscle (14 days). Arrows give median values and horizontal bars 95% confidence limits.

with that induced in normal fibres by Neostigmine (Fig. 9A and B, hatched columns). After Neostigmine the median increased from 0.08 to 0.36. This is nearly the same as the value 0.35 obtained in the preparation with the longest conduction block (14 days, Fig. 9C, hatched columns). Fig. 10A shows an example of the lack of correlation in a normal fibre ($r=0.04$) and the positive correlation ($r=0.40$)

Table 4. Characteristics of m.e.p.p. Including Kendall coefficient of correlation (τ) between amplitude and 1/2 decay time at original soleus endplates. Number of muscles and of fibres indicated.

| | m.e.p.p. characteristics | | | | | |
|-------------------------|--------------------------|-----------------|-----------------|---------------------|------------------|-------------------|
| | No./sec | Rise time msec | Amplitude mV | ampl. vs. rise time | Number of fibres | Number of muscles |
| Normal soleus endplates | 1.38 ± 0.1 | 0.89 ± 0.02 | 0.38 ± 0.01 | 0.08 ± 0.01 | 36 | 4 |
| After 14 days blocked | 1.20 ± 0.14 | 1.43 ± 0.04 | 0.60 ± 0.03 | 0.36 ± 0.02 | 31 | 4 |

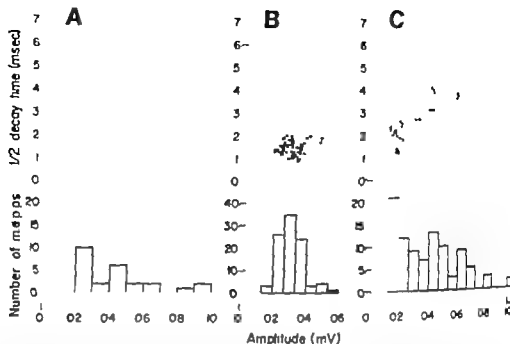


Fig. 5. Scatter diagram of 1/2 decay times vs. amplitude of individual m.e.p.p.s from a non-blocked fibular nerve junction (A) and 10 (B) days after cutting the soleus nerve and from a blocked fibular nerve junction 10 days after onset of paralysis (C) (upper row). Lower row shows distribution of m.e.p.p. amplitudes from the same 3 junctions.

probably depending on muscle activity in a graded manner.

Junctional AChE at original nerve impulse blocked synapses. In these preparations impulse conduction was blocked not only in the foreign nerve but also in the original nerve to the soleus muscle. It was therefore possible to examine how lack of impulse activity affected the maintenance of AChE at well established mature synapses.

Histochemistry. Histochemical staining for ChE did not give any clear indication of an effect of the nerve block. Both nerve impulse blocked and innervated original synapses stained well for ChE (Fig. 1 B, D) and neither differed notably from the staining of normal synapses. We therefore looked for more subtle changes by examining the m.e.p.p.s at the nerve impulse blocked synapses along the lines described above for the new synapses.

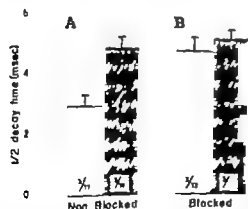


Fig. 6. Effect of Neostigmine on 1/2 decay time of m.e.p.p.s at ectopic junctions formed by non-blocked (A) and blocked (B) fibular nerves 13-15 days after onset of muscle paralysis. Open columns before and hatched columns after addition of 10^{-6} g/ml of Neostigmine to the perfusion fluid. Bars give S.E. of the mean. Number of muscles and of fibres is indicated.

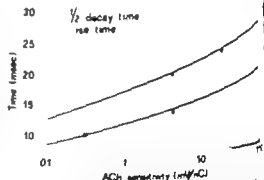


Fig. 7. Positive correlation between m.e.p.p. 1/2 decay time (●) or rise time (○) and extrajunctional ACh sensitivity. The fibres from which the m.e.p.p.s were obtained in the 10 mM ACh were generated at junctions formed by blocked m.e.p.p.s in a preparation where some of the m.e.p.p.s had already recovered from the block starting 1 day earlier. Kendall's correlation coefficients were 0.74 (1/2 decay time) and 0.83 (rise time).

c. Amplitude of m.e.p.p. at original soleus junctions ("old") and ectopic junctions formed by fibular (new) before and after exposure to 10^{-6} g/ml of Neostigmine

of the nerve indicated

| | m.e.p.p. amplitude mV | | | |
|------------------------|-----------------------|-------------------|--------------|------------------|
| | Before Neostigmine | After Neostigmine | (%) increase | Number of fibres |
| control | | | | |
| less nerve not blocked | 0.41 ± 0.14 | 0.61 ± 0.14 | 49 | 9 |
| less nerve blocked | | | | |
| 1-15 days | 0.50 ± 0.13 | 0.53 ± 0.14 | 6 | 15 |
| control | | | | |
| nerve not blocked | 0.34 ± 0.07 | 0.40 ± 0.10 | 18 | 11 |
| nerve blocked | | | | |
| 1-15 days | 0.49 ± 0.14 | 0.44 ± 0.14 | -10 | 13 |

Synapse formation in the absence of nerve impulse activity

A cuff around the sciatic nerve blocked impulse activity both in the original nerve to the soleus and in transplanted fibular nerve. The first block was necessary to paralyse the soleus and render it receptive to innervation by the fibular nerve (Janet et al. 1973). A similar effect could have been achieved by cutting the original nerve to denervate the muscle (Fox & Thesleff 1967). The second block prevented nerve impulses from being conducted into the distal 1.2 cm of the transplanted nerve fibres and allowed us to ask if this would affect the ability of the nerve to form synapses. The finding was that blocked nerves innervated approximately the same percentages of muscle as unblocked nerves did in denervated muscles. Furthermore the synapses developed in much the same way in the two preparations except that little junctional AChE appeared in the blocked junctions (see below). This shows that neither nerve impulse activity evoked transmitter release nor evoked muscle activity is essential for the formation of functional neuromuscular connections. It extends to a mammalian *in vivo* preparation previous reports of synapse formation in the presence of impulse blocking agents in tadpoles (Harrison 1964) and *in vitro* from explants of rat spinal cord and L6-myotubes (Kadokoro et al. 1976). Bennett & Slater (1980) reported that high and stable ACh sensitivity develops underneath surgically exposed foreign nerve terminals indicating that the action of this post-junctional specialization does not require impulse activity. The present work is consistent with this and indicates in addition that

presynaptic structures for transmitter release also form in the absence of impulse activity.

While the essential events in synapse formation clearly occur in the absence of impulse activity our tests do not exclude more subtle effects of impulse activity on for example efficiency of transmission or on number and distribution of new synapses on single fibres.

Condition block by nerve cuffs

Criticism has been raised against the use of cuffs for blocking nerve impulse conduction (Bunt & Vrbová, 1975). The mechanism behind the block is not clear and the cuffs may have different unknown effects.

In these experiments we used plain silicone cuffs containing no local anaesthetics or other drugs. The block developed rapidly about 20-30 h after application of the cuff in contrast to the immediate block caused by cuffs containing local anaesthetics. The internal diameter of the cuff was critical for successful block and variations of 1.2 mm made the block either ineffective or caused extensive nerve degeneration. It is known that compression may lead to local demyelination (Ochoa et al. 1971) which by itself blocks impulse conduction (Rusakovskiy & Sears 1972). It seems likely that the cuff blocked impulse conduction by compressing the nerve.

The delayed onset of the conduction block may be attributed to reactive oedema within the cuff which could gradually increase the compression. The fact that injection of cortisone prevents the block provides evidence for such a mechanism (Carpino & Luxenberger unpublished). It is interesting that the immediate block caused by cuffs containing local anaesthetic is sometimes transient and that a second long-lasting block develops about 20-30 hours after application of the cuff (Carpino et al. 1977). Also with cuffs containing anaesthetics the internal diameter is critical. It is likely that these cuffs block impulse conduction by causing an immediate but transient

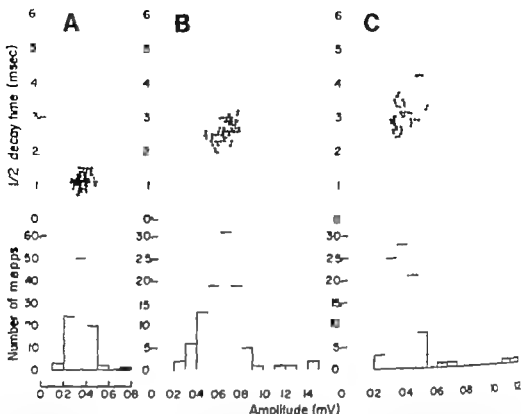


Fig. 10 Scatter diagram of $\frac{1}{2}$ decay times vs. amplitude of individual m.e.p.p.s from a normal soleus endplate before (A) and after (B) exposure to 10^{-6} g/ml of Neostigmine and from a soleus nerve endplate after a sciatic nerve block of 12 days duration (C). Lower row shows m.e.p.p. amplitude distributions from the same 3 endplates.

obtained in the same fibre 60 min after application of Neostigmine. Fig. 10C is from a fibre after 12 days of nerve conduction block. There is a positive correlation ($r=0.36$) resembling that found in Neostigmine treated normal fibres (Fig. 9B).

Effect of Neostigmine. Experiments with Neostigmine produced perhaps the clearest evidence

that the conduction block reduces AChE activity at mature endplates. In the impulse blocked preparation Neostigmine increased only moderately (22%) the $\frac{1}{2}$ decay time of m.e.p.p.s already abnormal as a result of the paralysis (Fig. 11B). In normal preparations, on the other hand, the increase was much larger (86% Fig. 11A). It is possible that these differences would have been even larger in these experiments with Neostigmine because the same high room temperature as the previous group of expts (see Methods). A low temperature is known to prolong m.e.p.p. time course and to reduce the prolonging effect of esterase inhibitors (Boyd & Martin 1956).

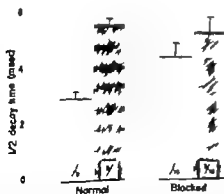


Fig. 11 $\frac{1}{2}$ decay times of m.e.p.p.s at original soleus endplates before (open columns) and after (hatched columns) application of 10^{-6} g/ml of Neostigmine in normal muscles and in muscles paralysed for 13-15 days by a chronic sciatic nerve block. Bars give \pm S.E. of the mean. Number of muscles and of fibres is indicated.

DISCUSSION

This work deals in particular with two aspects of neuromuscular synapse formation: (1) the ability of nerves to form synapses in the absence of impulse conduction to the nerve terminals and evoked muscle activity, and (2) the induction of AChE at newly formed endplates and the maintenance of AChE at established mature synapses.

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block due to the local anaesthetic and a later prolonged block due to compression.

Whatever the exact mechanism behind the block it is clear that blocked nerve fibres do not differ in any fundamental way from intact nerve fibres with respect to conduction of impulses distal to the block or capacity to form new functional synapses. The competence of the nerve in these respects argues against serious injurious effects of the cuffs used in the present experiments.

Impaired formation of AChE at new synapses

The nerve conduction block prevented the appearance of AChE at the developing new synapses almost completely. This result is consistent with other evidence that formation of junctional AChE requires evoked muscle activity (Lomo & Slater 1980b). By themselves however the present experiments do not distinguish between neural and muscular controlling factors because the cuffs may not only interfere with muscle activity but possibly also with axonal transport of neurotrophic substances controlling AChE formation (Fernandes & Inestrosa 1976; Younkin et al. 1978) and with the transport and release of AChF itself (Skau & Brimjoin 1978). The low AChE activity present at some impulse blocked synapses may be taken as evidence of an impulse independent control mechanism. However this marginal activity may also be accounted for by fibrillatory activity known to develop in nerve blocked rat muscles (Lomo & Rosenthal 1977). Fibrillatory activity has been shown to lower slightly denervation hypersensitivity (Purves & Sakmann 1974). The same muscle activity could by virtue of its reciprocal effects on extrajunctional ACh sensitivity and junctional AChE activity (Lomo & Slater 1980b) lead to the formation of small amounts of junctional AChE. Finally the possibility cannot be excluded that some undetected impulse activity may arise in these preparations from temporarily failing conduction blocks or impulse generation near the site of compression (Howe et al. 1978).

Reduction in AChE at mature endplates

Nerve impulse activity was shown to be important not only for the formation of AChE at new synapses but also for its maintenance at old synapses. The electrophysiological evidence indicated a major re-

duction in AChE acting on nerve released ACh ("functional AChE") at impulse blocked endplates. The sensitivity to Neostigmine was markedly reduced and in a few fibres completely absent.

The major reduction in "functional AChE" is in contrast to the considerable histochemically detectable ChE activity at both denervated and nerve blocked endplates. This raises the possibility that some of the endplate Cholinesterases may not actually act on nerve released ACh. Such a possibility may not seem unreasonable considering the different location of ChE esterases relative to ACh receptors (Fertuck & Salpeter 1976): the very long persistence after denervation of some forms of ChE relative to the rapid disappearance of the 16S form found mainly at endplates (Hall 1973; Vigny et al. 1976) and finally the reported persistence after denervation of histochemically detectable ChE as affecting focally applied ACh (Pérot-Dechanet 1968).

The reduction in "functional AChE" shown here is consistent with results recently obtained by Miller et al. (1978). These authors assayed total ChE of endplate regions biochemically and showed that chronic nerve impulse block by tetrodotoxin had the same effect as denervation on ChE activity at soleus muscle endplates.

In conclusion these and previous results (Lomo & Slater 1978 and 1980a and b) suggest the following roles of muscle activity in synapse formation: in the absence of activity muscle becomes insensitive. New synapses may then form independent of nerve and muscle activity. The new innervation reestablishes muscle activity which causes extrajunctional ACh sensitivity to disappear and junctional AChE to appear and the muscle in turn becomes refractory to further innervation.

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Non-autonomic component in bradycardia of endurance trained men at rest and during exercise

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LEWIS S F, NYLANDER E, GAD P & ARESKOG N H. Non-autonomic component in bradycardia of endurance trained men at rest and during exercise. *Acta Physiol Scand* 1980; 109: 297-305. Received 11 Nov 1979. ISSN 0001-6772. August Krogh Institute, University of Copenhagen, Denmark and Department of Clinical Physiology, Regionalläkhuset, Linköping University, Sweden.

Autonomic nervous alterations have generally been held responsible for the bradycardia of the endurance athlete. In order to determine whether there is also a non-autonomic component in the bradycardia of long-term training we compared the intrinsic heart rate (HR) of highly trained bicyclists (heart volume: 995 ± 155 ml) with that of untrained men (heart volume: 805 ± 193 ml) at rest and during bicycle ergometer exercise at 40, 75 and 100% of maximal oxygen uptake ($\dot{V}_{O_{2max}}$). Intrinsic HR was achieved by combined vagal and beta-adrenergic blockade with atropine and propranolol or metoprolol (cardioselective) injected intravenously. Intrinsic HR was significantly lower in trained (T) than in untrained (UT) at rest and at all levels of exercise. The chronotropic reserve from resting HR to maximal HR was identical in the two groups. Nearly identical intrinsic HRs were achieved with atropine and either beta-adrenergic antagonist. HR differences between T and UT were very similar in magnitude—approximately 13 beats/min—at rest and during exercise at given percentage of $\dot{V}_{O_{2max}}$, with and without autonomic blockade. Evidence is thus provided for a non-autonomic component in the bradycardia of well-trained men which may be responsible for parallel downward shift in the relationship between HR and percentage of $\dot{V}_{O_{2max}}$. The lower intrinsic HR in well-trained men might be explained by i.e. the cardiac enlargement.

Key words: Physical training, bradycardia, autonomic nervous blockade, propranolol, metoprolol.

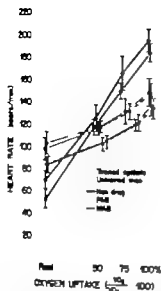
One of the hallmarks of the endurance athlete is a slower heart rate (HR) at rest and during exercise. The mechanisms involved are still controversial (Scheuer & Tipton 1977). Most studies focused on the role of the autonomic nervous system in the training bradycardia (Bravadin & Cerretti 1975; Ekblom et al. 1973; Winder et al. 1975). However it also has been proposed that a non-autonomic component, i.e. a lower intrinsic heart rate, may be involved (Badeer 1975; Sutton et al. 1975). Animal studies employing a wide variety of techniques provide findings in favour of (Barnard et al. 1973; Botter et al. 1973; Hughson et al. 1977; Sigmond et al. 1977) and opposed to (Porspark & Tipton 1970; Tipton et al. 1977) a lower intrinsic heart rate after training. In man combined parasympa-

thetic and beta-adrenergic blockade with atropine and propranolol has been used to study intrinsic HR at rest and during exercise (Jose & Collison 1970; Jose et al. 1970; Nordenfeldt 1971; Sutton et al. 1967). We recently demonstrated reduced intrinsic HR at a given submaximal workload, but not at rest or maximal exercise after short-term endurance training in man (Lewis et al. 1980).

The purpose of the present study was to examine the non-autonomic component of the bradycardia at rest and during submaximal and maximal exercise after very intense long-term endurance training.

This study was performed while Steven Lewis was George C. Marshall Fellow. His present address is: Division of Cardiology, University of Texas, Southwestern Medical School, Dallas, Texas, 75235 USA.

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Heart rate in trained and untrained at rest and during exercise requiring approximately 50, 75 and 100% maximal oxygen uptake without treatment and with autonomic blockade. Mean \pm 1 S.D. PAB = Propranolol + atropine blockade. MAB = Metoprolol + atropine blockade.

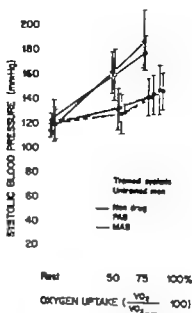


Fig. 2 Systolic blood pressure at rest and during submaximal exercise in trained and untrained without treatment and with autonomic blockade. Mean \pm 1 S.D. Abbreviations as in Fig. 1.

for repeated measures are performed to determine statistical significance of observed differences among the treatments within each group. If significant differences between each pair of test means are compared with Dunnett-New Multiple Test (Snedecor & Cochran). For all tests, $P < 0.05$ was accepted as significant. Correlation coefficients were calculated by the method of least

RESULTS

athletes had significantly larger heart volumes than the untrained men, 995 ml vs. 805 ml ($P < 0.05$) (Table 1). The heart volumes per m^2 body surface and per kg body weight were also significantly larger in the trained than in the untrained group, 540 ml/ m^2 ($P < 0.05$) and 15.0 vs. 11.5 ml/kg ($P < 0.01$), respectively. In the trained and untrained men combined ($n = 16$) there was a negative correlation between the heart volume in ml/ m^2 BSA and the resting HR without blockade ($R = -0.64$) after PAB and MAB ($R = -0.41$ and -0.43 respectively).

HR was lower in the T vs. the UT group at rest and at all levels of exercise up to

maximal (Fig. 1). HR was 53 vs. 70 beats/min ($P < 0.05$) at rest, 118 vs. 126 beats/min at 50% V_{max} , 150 vs. 166 beats/min at 75% V_{max} ($P < 0.05$) and 184 vs. 195 beats/min at V_{max} ($P < 0.05$) in the T and UT groups respectively.

After combined parasympathetic and beta adrenergic blockade with atropine and propranolol HR was also lower in the T vs. the UT group at rest and at all levels of exercise up to maximum (Fig. 1). HR was 84 vs. 103 beats/min at rest, 105 vs. 118 beats/min at 50% V_{max} , 122 vs. 132 beats/min at 75% V_{max} and 135 vs. 148 beats/min at V_{max} in the T and UT groups, respectively ($P < 0.05$ for all comparisons). Very similar HR results were obtained after combined autonomic blockade with atropine and metoprolol (Fig. 1). Maximal HR was 46 and 49 beats/min lower after autonomic blockade with each beta-adrenergic antagonist in the T and UT groups respectively (Fig. 1).

Systolic blood pressure

Systolic blood pressure was lower in the trained than in the untrained men at rest (114 vs. 125 mmHg, respectively, $P < 0.05$). However this difference was not present after autonomic blockade

Table 1 Physical characteristics of subjects

Mean \pm 1 S D

| Group | Age (yr) | Height (cm) | Weight (kg) | Heart volume (ml) | V _{max} (mmol kg ⁻¹ min ⁻¹) |
|----------------------|------------|--------------|-------------|-------------------|---|
| Sedentary men (n=8) | 26 \pm 4 | 181 \pm 11 | 70 \pm 8 | 805 \pm 195 | 2.0 \pm 0.3 |
| Elite cyclists (n=8) | 21 \pm 7 | 177 \pm 4 | 67 \pm 5 | 995 \pm 153 | 3.4 \pm 0.2 |

This was accomplished by comparing the intrinsic HR of highly trained competitive bicyclists with that of untrained men. Parasympathetic blockade was achieved with atropine and beta adrenergic blockade with propranolol, a non-cardioselective antagonist or metoprolol, a cardioselective antagonist, in order to determine whether the results of previous intrinsic HR studies may have been affected by the exclusive use of propranolol as beta-blocker.

METHODS

Subjects

8 elite long-distance bicyclists (trained=T) and 8 sedentary men (untrained=UT) volunteered as participants in this study. All the elite cyclists had been bicycling 450–700 km/week for 5–11 years prior to testing and had been cycling competitively for at least 5 years. The sedentary men were students or laboratory personnel who had never been competitive endurance athletes and who had not participated in regular training for at least one year immediately prior to the study. Physical characteristics of the subjects are given in Table 1.

Procedures

After a day of preliminary submaximal and maximal exercise tests, all subjects were tested on 3 separate days under the following conditions: (1) without drugs, (2) after i.v. injection of atropine sulfate plus propranolol hydrochloride, and (3) after i.v. injection of atropine sulfate plus metoprolol tartrate. Test days were at least one week apart in the sedentary group and in the cyclists no tests were administered for at least 4 days after one of the drug treatments. The conditions were administered in a random order. Each subject was tested at approximately the same time each day. The subjects fasted and abstained from smoking for at least 2 h prior to arriving at the laboratory and did not perform any strenuous exercise prior to being tested each day.

On each day resting HR was determined after at least 20 min rest in the supine position. On drug days the subjects remained supine and drug infusions were made through a catheter placed in an antecubital vein. First 0.04 mg/kg body weight atropine and then either 0.5 mg/kg propranolol, a non-selective beta-receptor antagonist or 0.3 mg/kg metoprolol, a cardio-selective beta-blocker without

intrinsic activity (Abbad et al. 1975) were injected over a total time of 4–5 min. The subject remained supine 1 min after completion of the propranolol or metoprolol injection prior to measurement of the resting heart rate. None of the subjects complained of anticholinergic side effects of the atropine during the tests.

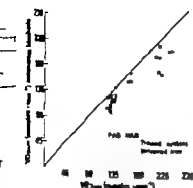
On each day the subjects then performed 3 exercise tests on a bicycle ergometer in the upright position. The first test was performed for 6 min at a workload equal to approximately 50% of maximal oxygen uptake ($V_{O_{2max}}$) as determined by preliminary testing. After a 15 min rest, a second exercise test was performed for 6 min at a workload approximately equal to 75% $V_{O_{2max}}$. In the 3 experiments the subjects were given an additional dose of 0.05 mg/kg propranolol or 0.1 mg/kg metoprolol just prior to the second submaximal test. Following the second maximal test the subjects rested for 10 min. $V_{O_{2max}}$ was then determined with stepwise increased workload until exhausted the subjects within 4–6 min.

Expired air was collected in Douglas bags during the last 2 min of submaximal exercise and the last 3 min of maximal work. HR was recorded continuously during exercise. Fingertip blood for determinations of lactate concentration was obtained at rest, 2 min after each maximal exercise and 5 min after maximal rest. Systolic blood pressure was obtained by the cuff method at rest on the bicycle ergometer immediately before beginning each submaximal test and during the fifth min of submaximal work.

The sedentary group was tested on a Knapik bicycle ergometer and the elite cyclists were tested on an E. bicycle ergometer (type EM369). Expired air volume was measured with a balanced spirometer or gasometer, percentages of O₂ and CO₂ in expired air were determined by the Scholander method or by a Servomex O₂ analyser Beckman model LB-2 CO₂ analyzer which were checked regularly by the Scholander method. Oxygen uptake was calculated according to standard procedures. HR was determined from ECG tracings. Blood lactate concentrations were determined by fluorometric and spectrophotometric methods (Jorfeldt & Juhlin-Danefelt 1973; dahn 1959).

The chest X-rays were obtained in the standing position with a film-focal distance of 1.5 m. The heart volume was determined by one radiologist according to L. (1939). The relative heart volume was calculated by division of the total heart volume to the body surface according to DuBois formula.

Between the groups statistical comparisons were made by t-tests for non-paired observations and by analysis of variance for paired observations.



Maximal oxygen uptake ($\dot{V}O_2$ max) achieved in the presence of autonomic blockade compared to $\dot{V}O_2$ max in the resting state in trained and untrained men. (45 min, 1 l/min.)

blood lactate was lower ($P < 0.05$) after autonomic blockade with each beta-blocker than in untrained conditions (when however the per cent work load was considerably higher). In the untrained blood lactate at 75% $\dot{V}O_2$ max and after maximal exercise was higher ($P < 0.05$) after metoprolol and atropine than after propranolol and atropine.

DISCUSSION

Endurance cyclists exhibited several of the well-known characteristics of endurance training—a lower heart rate at rest and during exercise than the untrained men. Heart volume was larger in T than in UT—a difference similar in magnitude to that reported previously between untrained controls and highly trained bicyclists (Sahlin 1967) or well trained runners (Häkkinen 1979). Unfortunately the X-rays for heart size determinations for technical reasons had to

be done in the standing position. In the lying position, it is not unlikely that the differences between T and UT had been still higher.

A lower maximal HR in endurance athletes than in sedentary controls has been observed in some studies (Saltin & Åstrand 1967) but not others (Pollock 1977). In the present study maximal HR was lower in the very homogenous group of trained men than in the untrained group. However the cardiac chronotropic reserve of the trained men was not diminished as their resting HR was also correspondingly lower (Fig. 1).

In man, the non-autonomic HR (also referred to as the intrinsic HR) (Sutton *et al.* 1967) has been studied by pharmacological blockade with competitive parasympathetic and beta-adrenergic antagonists (Jose & Collison 1970, Jose *et al.* 1970, Nordenfeldt 1971). The question can always be raised whether or not such blockades are complete. In the present study 1 v doses of 0.04 mg/kg atropine and 0.5 mg/kg propranolol or 0.5 mg/kg metoprolol were administered initially. At this dosage level additional doses of atropine or propranolol will not alter submaximal exercise HR (Jose *et al.* 1970, Lewis *et al.* 1960). Nevertheless, to ensure complete blockade we added a small additional dose of beta-blocker prior to the second submaximal exercise test. This did not further lower the exercise HR, indicating a complete blockade.

The effects of a cardioselective (metoprolol) and a non-selective (propranolol) beta-blocker (MAB and PAB respectively) included in the autonomic blockade were in most respects not significantly different. HR at rest and at all level of exercise was the same after PAB and MAB. The reduction of almost 50 beats/min in HR during maximal work after PAB and MAB reflects the equipotency of the two beta-blockers in the doses given (Johansson

Table 3. Blood lactate mmol/l

Table 3 shows blood lactate concentration (mmol/l) during exercise with and without autonomic blockade. Significant ($P < 0.05$) difference between non-drug treatment and Propranolol and Atropine treatment. ** and †† $P < 0.01$.

| | Group | Non-drug | Propranolol atropine | Metoprolol + atropine |
|-----|-------|--------------|-------------------------|--------------------------|
| max | T | 0.91 ± 0.69 | 1.00 ± 0.28 | 1.15 ± 0.84 |
| | UT | 1.18 ± 1.4 | 2.13 ± 0.86 | 2.30 ± 0.95 |
| 75% | T | 1.84 ± 0.79 | 1.95 ± 1.99* | 4.04 ± 1.77 †† |
| | UT | 4.90 ± 1.46 | 5.80 ± 1.89 | 5.77 ± 1.86 |
| | T | 9.28 ± 1.46 | 9.01 ± 1.55 | 6.70 ± 1.43 †† |
| | UT | 11.70 ± 1.65 | 9.09 ± 2.49* | 9.36 ± 1.91 †† |

Table 2 Oxygen uptake (l min^{-1}) pulmonary ventilation (l min^{-1}) and \dot{V}_E/\dot{V}_O and Mean \pm 1 S D during exercise with and without autonomic blockade. Significant difference ($P < 0.05$) between non-treatment and Propranolol and Atropine treatment or \dagger Metoprolol and Atropine treatment. and $\ddagger P < 0.01$

| | Group | Non-drug | Propranolol + atropine | Metoprolol + atropine |
|-----------------------------|-------|------------------|--------------------------|-------------------------------|
| 50% \dot{V}_O max | T | 108 \pm 12 | 105 \pm 1 [†] | 106 \pm 1 [†] |
| | UT | 70 \pm 9 | 65 \pm 9 | 67 \pm 10 [†] |
| 75% \dot{V}_O max | T | 168 \pm 6 | 160 \pm 20* | 165 \pm 4 |
| | UT | 104 \pm 17 | 100 \pm 18 | 98 \pm 13 [†] |
| \dot{V} max | T | 226 \pm 21 | 178 \pm 20 | 189 \pm 18 [†] |
| | UT | 139 \pm 16 | 111 \pm 16 | 126 \pm 13 [†] |
| \dot{V}_E 50% max | T | 5.5 \pm 5.8 | 51.5 \pm 7.6 | 51.7 \pm 6.9 |
| | UT | 47.2 \pm 7.7 | 41.0 \pm 6.6 | 43.5 \pm 6.5 [†] |
| \dot{V} 75% max | T | 84.8 \pm 13.6 | 92.8 \pm 17 | 98.7 \pm 14 [†] |
| | UT | 77.8 \pm 13 | 71.0 \pm 15 | 70.2 \pm 11.1 |
| \dot{V} max | T | 151.5 \pm 11.5 | 115.5 \pm 10.5 | 117.0 \pm 17.6 [†] |
| | UT | 138.6 \pm 15.6 | 108.0 \pm 11.8 | 114.3 \pm 13.3 [†] |
| \dot{V}/\dot{V}_O 50% max | T | 0.49 \pm 0.03 | 0.48 \pm 0.05 | 0.49 \pm 0.04 |
| | UT | 0.68 \pm 0.11 | 0.63 \pm 0.09 | 0.66 \pm 0.11 |
| \dot{V}/\dot{V} 75% max | T | 0.50 \pm 0.04 | 0.58 \pm 0.06 | 0.59 \pm 0.06 ^{††} |
| | UT | 0.70 \pm 0.09 | 0.71 \pm 0.10 | 0.77 \pm 0.08 |
| \dot{V}/\dot{V} max | T | 0.67 \pm 0.05 | 0.65 \pm 0.04 | 0.67 \pm 0.07 |
| | UT | 1.00 \pm 0.11 | 0.90 \pm 0.08* | 0.91 \pm 0.09 |

(Fig. 2) During submaximal exercise there was no significant difference in the systolic blood pressure between the trained and untrained men with and without autonomic blockade. Systolic blood pressure was significantly lower in each group during exercise after autonomic blockade by the same amount after PAB and MAB (Fig. 2).

Pulmonary ventilation

Differences in \dot{V}_E max between the T and UT groups were not significant under any exercise condition (Table 2). \dot{V}_E max was reduced under both autonomic blockade conditions in the T and UT groups ($P < 0.05$). In each group there was no significant difference in \dot{V}_E max between the different drug combinations (Table 2). After autonomic blockade \dot{V}_E was slightly lower in the UT group at 50% \dot{V}_O max ($P < 0.05$) and higher in the T group at 75% \dot{V} max ($P < 0.05$) compared to the \dot{V}_E for each group unblocked.

The quotient between \dot{V} and \dot{V}_E was unchanged with and without autonomic blockade in T and with and without the metoprolol atropine blockade in UT at maximal work load. It was higher in UT at all levels of exercise implying an excess ventilation in this group. In both groups the quotient tended to be higher at max work load.

Oxygen uptake

\dot{V}_O max was 3.4 vs. 2.0 mmol/kg/min in T and UT groups respectively ($P < 0.05$) (Table 1). After atropine and propranolol administration a greater reduction of \dot{V}_O max was observed in the T than in the UT (21 vs. 13% respectively $P < 0.05$) (Table 1 and Fig. 3). \dot{V}_E max was 16 and 10% lower ($P < 0.05$) in the T and UT groups respectively after atropine and metoprolol administration (Table 2 and Fig. 3). During submaximal work \dot{V} was slightly (3.4 vs. 3.1 mmol/min) but significantly lower after autonomic blockade in both groups (Table 2).

Blood lactate

Blood lactate was lower ($P < 0.05$) in the T than in the UT group under each condition after exercise at 50% \dot{V}_O max (Table 3). Similar results were obtained after exercise at 75% \dot{V}_O max and during maximal exercise except that there were no significant differences between T and UT after metoprolol plus atropine administration.

After exercise at 75% \dot{V} max in the T and UT groups blood lactates tended to be higher after autonomic blockade with each beta-blocker than under normal conditions although significantly higher blood lactates were obtained only in the T group (Table 3). After maximal exercise in the T and UT groups blood lactates were higher in the T than in the UT group.

1977). However the question of whether or not there is a non-autonomic component, i.e. a low-intrinsic HR, in the training bradycardia remains undecided. In comparisons of trained and untrained subjects, a lower cardiac rate has been observed in isolated sinoatrial preparations (Bolter et al. 1973; Nott et al. 1977) but not isolated whole hearts (Jorgensen & Schaefer 1970; Tipton et al. 1977) of cats, in trained rats after pithing and denervation (Sigvardsson et al. 1977) and in trained rats (Nott et al. 1976) but not in trained dogs (Tipton et al. 1977) after combined atropine plus propranolol blockade. In man there has been no conclusive evidence that resting HR after full autonomic blockade is lower after a short period of training. This may be due to the fact that a prolonged and intensive training period is necessary to elicit the intrinsic HR adaptation that has been observed in animals. Training studies are ordinarily carried out over a much greater portion of an animal's lifespan than the lifespan of a man. We have circumvented the difficulties of a longitudinal study in by comparing a homogeneous group of highly trained endurance athletes with a group of untrained men.

Our data indicate that in humans there is also a lower intrinsic HR in trained than in untrained individuals. In the present study intrinsic HR at rest was a lower percentage of $V_{O_2 \text{ max}}$ was lower in T than in UT. This finding was definitely not the result of T being younger than UT since intrinsic HR in man and other mammals decreases with age (Nott et al. 1976; Jose & Collins 1970). Furthermore, HR differences between T and UT were similar in magnitude—approximately 13 beats min^{-1} at rest and during exercise at a given percentage of $V_{O_2 \text{ max}}$ with and without autonomic blockade (Fig. 1). Thus, non-autonomic component in the bradycardia of well-trained men may be due to a parallel downward displacement of the $V_{O_2 \text{ max}}$ relationship (Fig. 1).

Significant positive correlations between resting and maximal HR with and without autonomic blockade (Fig. 4) suggest that a training-induced adaptation in the electrophysiology of the sinoatrial node could reset both the resting and maximal HR to a lower level. In support of this hypothesis, Tipton et al. (1977) found a high positive correlation between the spontaneous frequency and the maximal frequency response to norepinephrine in isolated sinoatrial preparations from trained and un-

trained rats. Bronson et al. (1976) observed a decreased maximal exercise heart rate and an increased duration of the right atrial monophasic action potential at rest after training in healthy men. They attributed this finding to an altered ionic balance between the inside and outside of the pacemaker cell membrane resulting in an intracellular potassium accumulation.

The non-autonomic component of the bradycardia of well-trained men may also be related to cardiac enlargement. Holmgren & Åstrand (1966) found a significant inverse correlation between maximal heart rate and heart volume in well-trained men and women. Using anthropometric formulas, Ekblom (1969) calculated that the reduction in maximal heart rate after 4 years of training in one subject could be accounted for solely on the basis of a greater heart volume. In the present study heart volume was considerably larger in T than in UT and we found an inverse relationship between heart volume and resting heart rate ($R = -0.64$, $P < 0.01$). Heart volume provides only an overall estimate of cardiac size giving no dimensional assessment of the various chambers. However, in endurance athletes an increased heart volume determined roentgenographically is associated with an increase in ventricular and atrial dilatation measured by echocardiography (Blahem et al. 1979). Although the sinoatrial pacemaker increases its firing rate in response to stretch (Jensen 1971) the tonic stretch which could occur with the atrial dilatation of prolonged endurance training might inhibit the acute stretch response and lower the intrinsic HR (Blomqvist & Lewis 1979).

The mechanisms by which endurance training-induced bradycardia is achieved are thus complex. Furthermore, animal experiments have shown that a functioning adrenergic nervous system is necessary for the development of a training-induced intrinsic HR decrease in rats (Sigvardsson et al. 1977).

In conclusion, the present data suggest that after very intense long-term endurance training there is an important non-autonomic component in the bradycardia at rest and at all levels of exercise. This is in contrast to the findings in our earlier study (Lewis et al. 1980) in which we observed no change in the intrinsic HR at rest and during maximal exercise after short-term endurance training, but in accordance with several training studies in animal. In the present study we circumvented the difficulties

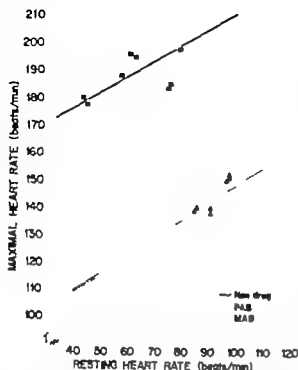


Fig. 4. Relation between maximal heart rate during exercise and heart rate at rest in the unblocked state and during autonomic blockade for the trained and the untrained group combined ($n=16$).

1975) and nearly or totally complete autonomic blockade even at this level of work (Lewis et al 1980). After PAB with smaller doses of propranolol Ekblom et al (1977) found a reduction of 38 beats/min in HR at maximal effort.

In the present study resting blood pressure was affected very little by PAB and MAB. With submaximal exercise the systolic blood pressure increase was smaller after autonomic blockade probably reflecting a lower cardiac output (Furberg 1968). Systolic blood pressures during exercise were very similar after PAB and MAB which may indicate that peripheral vascular effects which could be expected to be different in a cardio-selective and non-selective beta-blocker (Conolly et al 1976) are not the main determinants of blood pressure during exercise after combined vagal and beta adrenergic blockade using the present drug doses. At rest without autonomic blockade blood pressure was lower in T than UT in agreement with previous comparisons of athletes and non-athletes (Scheuer & Tipton 1977).

Another difference between a cardioselective and non-selective beta-blocker is the degree of bronchial constriction (Conolly et al 1976). However in the present subjects this was not reflected in the

pulmonary ventilation which was similar after UT and PAB during submaximal and maximal exercise. \dot{V}_E during submaximal exercise was also similar after MAB and PAB. Submaximal \dot{V}_E and \dot{V}_O however were in most cases slightly but significantly lower with than without autonomic blockade. However at the 75% max load in the trained group \dot{V}_E was higher with than without an autonomic blockade. Because of the large reduction in \dot{V}_E capacity after blockade in the trained men \dot{V}_E was near maximal for several of them.

The \dot{V}_O/\dot{V}_E work load tended to be lower after PAB and MAB than in the unblocked condition and the quotient between \dot{V}_E max and \dot{V}_O max was unchanged with and without autonomic blockade except for a lower quotient after PAB in the UT group (Table 3) which makes it unlikely that the blockades caused an increased work of breathing in the present subjects. Therefore it is also less conceivable that the ventilatory capacity was the limiting factor of the work capacity during PAB or MAB. \dot{V}_O max was lower after autonomic blockade in both groups. In each group the reduction in \dot{V}_O max after LAB tended to be greater than after MAB although this was not significant. After PAB and MAB the reduction in \dot{V}_O max was greater in the T group than in the UT group (after PAB 21 vs 13% respectively $P<0.05$). In a previous study (Lewis et al 1980) reduction in \dot{V}_O max after PAB was similar (18–19% 25–31 mmHg) before and after short term training.

The T subjects complained more of extreme fatigue in connection with maximal work than the UT subject. This could have been related to the greater reduction in blood lactate after maximal work during autonomic blockade in the T group (Table 3) which could probably explain the greater reduction in \dot{V}_E max in T than UT after autonomic blockade (Fig. 3). The latter results are in accordance with the findings of Furberg (1968) of a decreased working capacity in athletes compared to controls after propranolol blockade. He concluded that untrained subjects may compensate for a cardiac output decrease by an increased peripheral oxygen extraction while athletes already in the blocked state have a high a-v difference during heavy work and thus have a smaller reserve for compensation.

Most investigators have attributed the beneficial effects of endurance training to an increased vagal tone and a decreased beta-1 drive (Scheuer & Tipton 1977).

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of a long-term training study by comparing a group of elite athletes with a group of untrained men. Possible genetic differences between these two populations (Klissouras 1971) should be recognized and the conclusions therefore viewed with caution. The present data demonstrate too that the intrinsic HR at rest and during exercise is very similar when atropine is combined with either the non cardioselective beta-receptor antagonist propranolol or the cardioselective metoprolol.

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Local cerebral blood flow in the rat during severe hypoglycemia and in the recovery period following glucose injection

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ABDUL RAHMAN, A. AGARDH, C. D. & SJESJÖ B. K. Local cerebral blood flow in the rat during severe hypoglycemia, and in the recovery period following glucose injection. *Acta Physiol Scand* 1980; 109: 307-314. Received 13 Nov 1979. ISSN 0001-6772. Laboratory of Experimental Brain Research, E-Blocket, the Department of Neurosurgery and the Department of Internal Medicine, University of Lund, Sweden.

In order to assess the influence of severe hypoglycemia on local cerebral blood flow (l-CBF) artificially ventilated rats anaesthetized on 70% N₂O were injected with insulin to provide either an EEG pattern of slow-wave polyspikes, or cessation of spontaneous EEG activity for 5, 15 or 30 min ('coma'). In other animals, glucose was injected at the end of 30 min period of 'coma' and l-CBF was measured after recovery periods of 5, 30, 90, or 180 min. Local CBF was measured autoradiographically with ¹⁴C-iodoantipyrine as the diffusible tracer. In the slow-wave polyspike period l-CBF was increased in most of the structures studied, and reached values that were 1.4 to 3.2 times greater than control. In many structures, cessation of EEG activity was accompanied by a further increase in l-CBF, with some structures (thalamus, hypothalamus, pons, gray and cerebellar cortex) showing flow rates of 400-500% of control. The increase in l-CBF was unrelated to arterial hypertension, hypercapnia, or hypoxia. 5 min after glucose injection the hypertension persisted in only some of the structures studied, in others, the l-CBF were close to or below control values. During the subsequent recovery period l-CBF was markedly reduced with some structures (cerebral cortical areas, hippocampus, and caudate-putamen) showing flow rates of only 20-35% of control. In others, notably pons, gray and cerebellar cortex, secondary hypoperfusion was never observed. The hypoperfusion was unrelated to arterial hypotension, hypocapnia, or increase in intracranial pressure. It is concluded that like hypoxia and ischemia, substrate deficiency due to hypoglycemia is accompanied by vasodilatation in the brain. Furthermore, like long-lasting ischemia, severe hypoglycemia is followed by a delayed hypoperfusion syndrome that, by restricting oxygen supply, may well contribute to the final cell damage occurred.

Key words: Local cerebral blood flow, quantitative autoradiography, hypoglycemia.

It is reasonable to believe that the irreversible damage that may result from prolonged severe hypoglycemia is related to cellular energy failure. Thus, animal experiments have demonstrated that when blood glucose concentrations are reduced below normal, and spontaneous EEG activities cease, the tissue concentrations of phosphocreatine (PCr) and ATP are markedly decreased like those of ADP and AMP rise (Hänen et al 1974; Lewis et al 1974a; Norberg & Siesjö 1976). Associated with this change in cerebral energy state is a marked perturbation in concentrations of cyclic metabolites, citric acid cycle inter-

mediates and associated amino acids, probably largely reflecting oxidation of endogenous substrates (Lewis et al 1974b; Norberg & Siesjö 1976). It has recently been shown that such a state of pronounced energy failure can be upheld for 30 min and still allow extensive recovery of cerebral metabolic rate (Agardh et al 1978). However in the recovery period there was sustained increase in the lactate concentration and the lactate/

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Table 1 Physiological parameters and blood glucose during 1 min-induced hypoglycemia and in the 1 min period following glucose injection

The group consisted of 4 animals, control 10 animals. The values are mean \pm S.E. MABP=Mean arterial blood pressure

| | MABP (mmHg) | P _{aCO₂} (mmHg) | P _{aO₂} (mmHg) | pH | Temp (°C) | Blood glucose (μ mol g ⁻¹) |
|----------------------|------------------|--|---------------------------------------|------------------|----------------|---|
| W | 147 \pm 3 | 36.8 \pm 1.0 | 103 \pm 1 | 7.41 \pm 0.02 | 37.0 \pm 0.1 | 6.03 \pm 0.40* |
| Active poly- | 144 \pm 8 | 34.3 \pm 1.1 | 116 \pm 3 | 7.36 \pm 0.02 | 37.3 \pm 0.2 | 1.26 \pm 0.11 |
| EEG | | | | | | |
| Active EEG (mean) | 5 139 \pm 16 | 34.4 \pm 1.1 | 107 \pm 4 | 7.40 \pm 0.03 | 37.0 \pm 0.1 | 0.73 \pm 0.05 |
| | 15 151 \pm 7 | 34.1 \pm 1.2 | 103 \pm 2 | 7.34 \pm 0.04 | 37.0 \pm 0.3 | 0.66 \pm 0.12 |
| | 30 148 \pm 8 | 29.7 \pm 3.0* | 99 \pm 7 | 7.39 \pm 0.03 | 36.9 \pm 0.3 | 0.42 \pm 0.10 |
| Very (mean) after | | | | | | |
| in electrocortic EEG | 5 150 \pm 5 | 38.6 \pm 1.9 | 102 \pm 5 | 7.20 \pm 0.05 | 36.9 \pm 0.3 | 13.30 \pm 2.00 |
| | 30 143 \pm 10 | 37.0 \pm 1.9 | 114 \pm 1 | 7.25 \pm 0.02 | 37.1 \pm 0.1 | 6.94 \pm 0.52 |
| | 90 128 \pm 1 | 37.6 \pm 4.0 | 110 \pm 7 | 7.26 \pm 0.02* | 36.9 \pm 0.1 | 7.26 \pm 0.59 |
| | 180 111 \pm 7* | 37.3 \pm 4.4 | 101 \pm 7 | 7.19 \pm 0.05 | 37.0 \pm 0.2 | 8.58 \pm 0.61 |

S.E. for the 400 min control animals and the animal injected with insulin and infused with glucose (see text)
* $P < 0.01$ ** $P < 0.001$

period of ceased EEG activity and in which I-CBF measured either 5, 30, 90, or 180 min later. Glucose given in an initial dose of 0.5 ml of 50% (w/v) glucose, followed by constant infusion of the same at a rate of 1 ml/h. The values obtained were similar to those obtained in control animals maintained under otherwise identical conditions (Abdul-Rahman et al. 1979). However, since the hypoglycemic animals were maintained anesthetized for periods of 93–388 min, additional (uninjected) control animals were used which the period of anaesthesia was prolonged. Furthermore, in order to study whether or not anaesthetic doses administered had any influence on I-CBF, animals were injected with insulin (40 IU/kg), followed by the measurement of blood glucose concentrations where 5 μ mol/g by slow administration of a solution (1.0 ml of 50% w/v glucose solution in 0.9% saline). I-CBF was measured 400 min later. Other experiments the intracranial pressure (ICP) was recorded in cannula inserted in the cisterna magna during hypoglycemia in animals with an electrocortic recording for 30 min and following recovery for 180 min. The arterial blood pressure was simultaneously recorded from one femoral artery.

I-CBF was calculated as described in the previous section (Abdul-Rahman et al. 1979). Statistical differences between groups were calculated using Student's *t*-test.

RESULTS

Table 1 gives the body temperature, mean arterial pressure, arterial P_{aO₂}, P_{aCO₂} and pH as well as blood glucose concentrations during and after

hypoglycemia. 6 of the control animals were those previously reported (Abdul-Rahman et al. 1979). Since physiological parameters between these and the 4 animals studied presently (400 min of anaesthesia with or without insulin injection) were not obviously different, all 10 animals were pooled. The results of Table 1 shows that all hypoglycemic and recovery animals were normothermic and had mean arterial P_{aO₂} values of 100 mmHg, or higher. Arterial blood pressure was upheld except in the animals allowed recovery periods of 90 and 180 min in which moderate hypotension developed. Arterial P_{aCO₂} fell moderately after 30 min of ceased EEG activity but was close to normal in all other groups. Arterial pH was upheld during hypoglycemia but was reduced ($P < 0.001$) in the recovery groups.

Table 2 and Table 3 give I-CBF values measured in control, hypoglycemic, and recovery animals respectively. In order to facilitate discussion of results, Figs 1 and 2 show values in percent of control during (Fig. 1) and after (Fig. 2) hypoglycemia. As previously stated, I-CBF values for 6 of the 10 control animals was reported elsewhere but recalculated according to the autoradiographic standardization and used in the present report. In the two uninjected animals maintained under anaesthesia for 400 min, I-CBF values were within the range of these of the 6 original controls. The same was applied to one of the insulin-injected animals while the other had some I-CBF values slightly exceeding this range. However, since differences were small

pyruvate ratio and in spite of the fact that the cerebral cortical adenylate energy charge returned to normal values after 3 h the size of the adenine nucleotide pool remained below normal. We speculated that some cell damage must have been incurred, an assumption which has been borne out in a recent histopathologic study (Agardh et al. 1980; Kallimo et al. 1980).

In order to elucidate the mechanisms by which hypoglycemia leads to cell damage, it is important to establish whether energy failure due to substrate depletion is solely responsible or if circulatory factors cause a restriction of oxygen supply as well, adding a hypoxic insult to the primary one. During hypoglycemia this does not seem to be the case. Thus studies in man have shown that cerebral blood flow (CBF) is either normal or increased during hypoglycemic coma (Keely et al. 1948; Della Porta et al. 1964). In the rat CBF increased both in the slow wave polyspike period and during the first 15 min of hypoglycemic coma (Norberg & Siejö 1976). Furthermore calculations from substrate concentrations reveal that severe hypoglycemia is accompanied by an oxidation of cellular redox systems (Lewis et al. 1974b).

Less is known about circulatory events in the recovery period following glucose administration. After shortlasting coma both CBF and cerebral metabolic rate for oxygen (CMR_{O_2}) quickly return to normal values (Della Porta et al. 1964). In irreversible coma, though both CMR_{O_2} and CBF fall progressively (Fazekas et al. 1951). From the results published it is not possible to judge whether the fall in CBF was secondary to a reduced metabolic rate or the cause of it.

As reported in a preliminary communication we have recently observed that sustained hypoglycemia is followed by a progressive reduction in local CBF (I-CBF) during a 90 min recovery period (Siejö & Abdul Rahman 1979). The present communication provides data for I-CBF in hypoglycemia of sufficient severity to induce slow wave polyspikes in the EEG or cessation of EEG activity for 5, 15 or 30 min as well as in recovery periods of maximally 3 h following 30 min of hypoglycemic coma.

METHODS

1. Operative and sampling technique

Male Wistar rats, weighing 275–365 g, were starved for 24 h but had free access to tap water. They were then

injected with insulin (Novo Actrapid[®] Novo Industri A/S) in a dose of 40 IU/kg i.p. After 60 min the rats were anesthetized with 2.5–3% halothane. When spontaneous they were tracheotomized, connected to a Sjöling type respirator, paralyzed with tubocurarine chloride (0.5 mg/kg i.v.) and ventilated on 10% halothane in 70% N_2O . Respiration was adjusted to give arterial P_{CO_2} values of 35–40 mmHg. Body temperature measured in the rectum was kept close to 37°C by means of a heating bulb.

Both femoral arteries were cannulated, one for the pressure recordings and sampling of blood for analysis of pH, blood gases and glucose concentrations, the other for sampling of blood during I-CBF measurements. The latter catheter was cut to a length of 1.5 cm and inserted into the arterial ^{14}C -iodoantipyrine curve (see below) as well as measuring. One femoral vein was cannulated for infusion of ^{14}C -iodoantipyrine and the other one for infusion of glucose during recovery periods. Gold-plated screws were inserted into the skull bone and connected via leads to Elema EEG machine. When operative procedures were completed halothane supply was discontinued and the animals were ventilated on 70% N_2O and 30% O_2 .

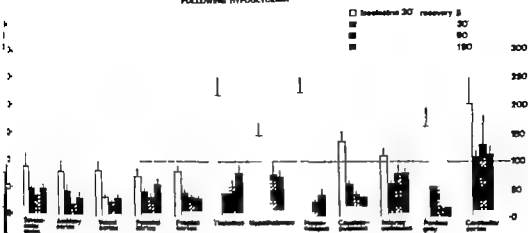
2. Measurement of local CBF

Local CBF was measured with quantitative autoradiography according to Sakurada et al. (1978). Details of procedure have been given in a preceding communication (Abdul Rahman et al. 1979). In summary, a solution containing ^{14}C -iodoantipyrine (New England Nuclear) at an activity of 40–50 μCi per rat was infused at a rate of 1 ml/min. In hypoglycemic animals, arterial blood samples were collected just before and every 3 s following start of infusion. After 30 s the animal was quickly decapitated and the head was frozen in Freon-1 at a temperature of minus 50–60°C. In recovery animals blood was sampled every 3 s and the period of infusion was 1 min. ^{14}C -activity in 50 μl blood samples was measured by liquid scintillation counting, while the activity in brain was assessed by applying 20 μm sections (at 10°C) to a film with subsequent macrodensitometric measurement. By the use of appropriate standards the tissue activity determined, allowing calculation of CBF in the skull shielded. A careful recalibration of the ^{14}C -activity showed that the values previously reported to be slightly in error. As a result, the present I-CBF values for control rats are about 10–15% higher than those previously published (Abdul-Rahman et al. 1979). Details of calculation procedures will be given in another publication (Ingvar, Abdul Rahman & Siejö 1980).

3. Experimental protocol and group

Animals were grouped according to the EEG pattern. One group, I-CBF was measured when the normal pattern had been transformed into one with slow polyspikes corresponding to a blood glucose concentration of $1.6 \pm 0.1 \mu mol/g$ (mean $\pm S.E.$). Three groups were studied in which spontaneous EEG had ceased for either 5, 15 or 30 min (blood glucose concentration of $1 \mu mol/g$ or lower). The last group contained animals in which EEG was present after

LOCAL CBF IN RECOVERY PERIOD FOLLOWING HYPOGLYCEMIA



Local CBF in the recovery period (5, 30, 90 or 180 min) following 30 min period of "isoelectric" hypoglycemia. Values are means \pm S.E. in percent of control.

sensorimotor cortex, hypothalamus, pons and cerebellar cortex. The inhomogeneous increase in flow between structures is illustrated by the fact that CBF in hippocampus and cerebellar vermis was 1.4- and 3.2-fold control respectively.

After 5, 15 and 30 min of severe hypoglycemia (with ceased EEG activity) I-CBF was increased in all structures studied with no obvious relationship to the period of hypoglycemic coma. In most structures the increase in I-CBF was similar to that

c) Local CBF ($\text{ml g}^{-1} \text{min}^{-1}$) during recovery period following glucose injection after a isoelectric of 30 min duration. Values are means \pm S.E.

| Structures | Control 10 | Isoelectric EEG 30' and recovery 5' =4 | Isoelectric EEG 30' and recovery 30' =4 | Isoelectric EEG 30' and recovery 90' =4 | Isoelectric EEG 30' and recovery 180' =4 |
|---------------------------------------|-----------------|--|---|---|--|
| Superficial cerebral structure | | | | | |
| Somatosensory | 1.57 \pm 0.09 | 1.44 \pm 0.34 | 0.77 \pm 0.06** | 0.55 \pm 0.09** | 0.77 \pm 0.11 |
| Auditory | 2.23 \pm 0.19 | 1.79 \pm 0.46 | 0.95 \pm 0.24*** | 0.44 \pm 0.02** | 0.69 \pm 0.20*** |
| Visual | 1.35 \pm 0.06 | 1.22 \pm 0.29 | 0.47 \pm 0.04** | 0.32 \pm 0.02** | 0.42 \pm 0.06** |
| Frontal | 1.30 \pm 0.07 | 1.10 \pm 0.20* | 0.69 \pm 0.04** | 0.53 \pm 0.09** | 0.88 \pm 0.15 |
| Parietal | 1.32 \pm 0.07 | 1.08 \pm 0.11 | 0.58 \pm 0.05** | 0.47 \pm 0.07** | 0.44 \pm 0.06** |
| Deep cerebral structure | | | | | |
| Thalamus | 1.30 \pm 0.06 | 1.90 \pm 0.41 | 1.10 \pm 0.11 | 0.70 \pm 0.14** | 1.02 \pm 0.16 |
| Hypothalamus | 0.98 \pm 0.05 | 1.44 \pm 0.21** | 0.97 \pm 0.14 | 0.75 \pm 0.24 | 0.71 \pm 0.12* |
| Hippocampus | 1.00 \pm 0.04 | 37 \pm 0.26** | 0.49 \pm 0.06** | 0.27 \pm 0.03** | 0.40 \pm 0.10* |
| Medial thalamus | 1.41 \pm 0.07 | 1.86 \pm 0.24 | 0.85 \pm 0.06** | 0.58 \pm 0.07** | 0.51 \pm 0.06** |
| Mid brain and pons | | | | | |
| Inferior colliculus | 2.28 \pm 0.05 | 2.76 \pm 0.32 | 1.37 \pm 0.1 | 1.78 \pm 0.38 | 1.80 \pm 0.15** |
| Pons grey | 1.10 \pm 0.04 | 1.97 \pm 0.37* | 1.13 \pm 0.08 | 0.91 \pm 0.22 | 0.85 \pm 0.14 |
| Cerebellum | | | | | |
| Cerebellar cortex | 1.03 \pm 0.05 | 2.09 \pm 0.47* | 1.13 \pm 0.10 | 1.35 \pm 0.52 | 1.16 \pm 0.14 |

* $P < 0.01$ ** $P < 0.001$

LOCAL CBF IN HYPOGLYCEMIA

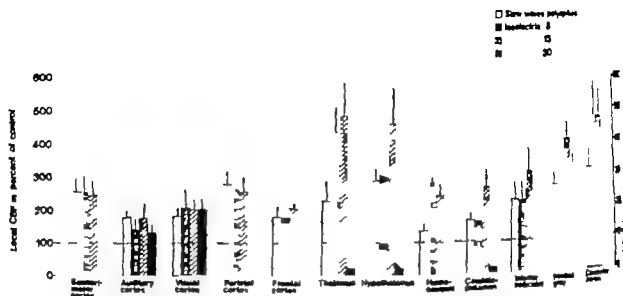


Fig. 1. Local CBF during insulin-induced hypoglycemia in animal showing an EEG pattern of slow wave polyspike cessation of spontaneous EEG activity ('isoelectric') for 5, 15 or 30 min. The values are means \pm S.E. in percent control.

all 10 animals were pooled to yield the control group of Table 1.

1-CBF during hypoglycemia

Table 2 and Fig. 1 demonstrate that during the period of slow wave polyspike 1-CBF was significantly

increased ($P < 0.01$) in all structures studied except hippocampus ($P < 0.05$). Absolute flow not exceeding $4 \text{ ml g}^{-1} \text{ min}^{-1}$ were observed in parietal, sensorimotor and auditory cortex and in superior colliculus. In relative terms, flow rates exceeding 2.5 times control were observed in parietal

Table 2. Local CBF ($\text{ml g}^{-1} \text{ min}^{-1}$) during insulin induced hypoglycemia.

The values are means \pm S.E.

| Structures | Control $n=10$ | Slow wave polyspike EEG $n=4$ | Isoelectric EEG 3 min $n=4$ | Isoelectric EEG 15 min $n=4$ | Isoelectric EEG 30 min $n=4$ |
|---|-------------------|-------------------------------------|-----------------------------------|------------------------------------|------------------------------------|
| A. Superficial cerebral structures | | | | | |
| Cortex | | | | | |
| 1 Sensorimotor | 1.57 ± 0.09 | 4.06 ± 0.62 | 4.00 ± 0.78 | $3.87 \pm 0.67^*$ | $1.54 \pm 0.09^{***}$ |
| 2 Auditory | 2.3 ± 0.18 | 4.01 ± 0.41 | 3.17 ± 0.75 | 4.50 ± 0.54 | $3.02 \pm 0.49^{***}$ |
| 3 Visual | 1.35 ± 0.06 | $4.0 \pm 0.17^*$ | 2.8 ± 0.70 | $2.50 \pm 0.18^*$ | $2.75 \pm 0.16^{***}$ |
| 4 Parietal | 1.50 ± 0.07 | 4.4 ± 0.58 | $4.00 \pm 0.78^*$ | 3.91 ± 0.64 | $3.79 \pm 0.41^{***}$ |
| 5 Frontal | 1.32 ± 0.07 | $3.6 \pm 0.17^*$ | 7.6 ± 0.76 | 4.75 ± 0.16 | $3.4 \pm 0.41^{***}$ |
| II. Deep cerebral structures | | | | | |
| 6 Thalamus | 1.30 ± 0.06 | 3.63 ± 0.76 | 5.67 ± 1.01 | $6.87 \pm 1.27^*$ | $3.88 \pm 0.77^{***}$ |
| 7 Hypothalamus | 0.98 ± 0.05 | 2.87 ± 0.35 | 3.02 ± 0.63 | 4.54 ± 1.01 | $3.76 \pm 0.17^{***}$ |
| 8 Hippocampus | 1.00 ± 0.04 | 1.38 ± 0.18 | $85 \pm 0.49^*$ | $3.93 \pm 0.98^*$ | $11 \pm 0.2^{***}$ |
| 9 Caudate-putamen | 1.41 ± 0.07 | 3.5 ± 0.31 | $90 \pm 0.69^*$ | $3.76 \pm 0.69^*$ | $3.11 \pm 0.17^{***}$ |
| C. Mid-brain and pons | | | | | |
| 10 Inferior colliculus | 2.8 ± 0.05 | 5.1 ± 1.23 | $5.06 \pm 1.79^*$ | 7.1 ± 1.67 | $3.70 \pm 0.54^{***}$ |
| 11 Pontine gray | 1.10 ± 0.04 | $2.96 \pm 0.38^*$ | 4.14 ± 0.66 | $3.60 \pm 0.99^*$ | $3.56 \pm 0.48^{***}$ |
| D. Cerebellum | | | | | |
| 12 Cerebellar cortex | 1.03 ± 0.05 | 3.5 ± 0.70 | 4.63 ± 1 | $4.85 \pm 0.89^*$ | $4.18 \pm 0.46^{***}$ |

$P < 0.5$ $P < 0.01$ $P < 0.001$

related to hypertension, hypercapnia, and as in previous results have failed to show intra- or extracellular bicarbonate concentrations are reduced (Lewis et al 1974b see also Møller et al 1978, Astrup et al 1978). In view of these results it appears very unlikely that cellular action of acids could have contributed to the coma. Since the increase in CBF was well expected already in the period of slow-wave sleep in the EEG a period characterized by only moderate elevation of extracellular K^+ (K_o) (Astrup & Norberg 1976) it is also unlikely to ascribe the hyperemia to a rise in K_o, although it appears probable that adenosine may be one of the coupling factors (Berne et al 1978). The mechanisms of the increase in CBF are at present unknown.

During the period of ceased EEG activity a few structures showed patchy perfusion. Since blood pressure remained normal it seems likely that some localized cell damage developed (cf. Auer et al 1973). If so, the coma was transient because in the 30 min following the flow again increased homogeneously. It should be emphasized that the secondary hyperemia during recovery developed at a time when the cerebral energy state is gradually improved and when lactate concentrations are elevated (Kjell et al 1978). Our results seem to exclude the possibility that the hypoperfusion was due to vasoconstriction. Thus, although the intracranial pressure increased during hypoglycemia, i.e. at the time of coma, this pressure was close to normal in the recovery period. The reduction in I-CBF occurred despite the fact that cerebral perfusion pressure was maintained at 100 mmHg, or higher, and at normal arterial CO₂ tension. These results suggest that cerebrovascular resistance was increased, although such an increase could be due to swelling of endothelial and/or surrounding parenchymal cells, or to vasoconstriction of small vessels. In view of the fact that circulatory changes are similar after prolonged ischemia (Kjell et al 1977) it would appear important to further explore the mechanisms responsible for the reductions in flow.

The present results demonstrate beyond any doubt that at least under the conditions of artificial ventilation and maintained arterial oxygenation the brain is not simply supplied with oxygen even at degrees of hypoglycemia that extinguish spontaneous EEG activity for up to 30 min. We conclude from these

results that the neuronal lesions that are observed at the end of a 30 min coma period (Agardh et al 1980, Kalimo et al 1980) are the result of substrate depletion alone and should not have been influenced by a concomitant restriction of oxygen supply.

The same conclusion obviously cannot be drawn with respect to the final lesions that result from severe hypoglycemia since I-CBF in some structures fell to about 25% of control in the recovery period. Our previous results showing a slow but relatively extensive recovery of cerebral energy state after 30 min of hypoglycemic coma (Agardh et al 1978) were obtained on cortical samples from frontoparietal regions. As the present results show CBF in these regions fell to about 30–40% of control after a 90 min recovery period. It seems reasonable to assume that this flow reduction retarded metabolic recovery. Possibly it could also have contributed to the final lesions due to the neuropathologic injury that is observed after long recovery periods (Agardh et al 1980, Kalimo et al 1980). However, if this is true one must suspect that metabolic recovery is less complete and cell damage more extensive in structures with an even greater reduction in CBF e.g. auditory and visual cortex. Clearly it seems highly warranted to further explore the relationship between local blood flow and metabolism as well as the extent of cell damage in various tissue structures.

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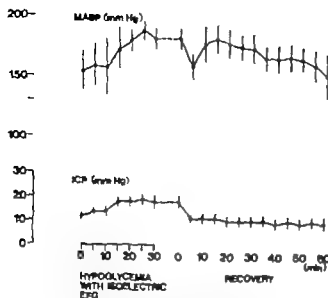


Fig 3 Mean arterial pressure (MABP) and intracranial CSF pressure during insulin induced hypoglycemia (isoelectric EEG for 30 min) and in the recovery following glucose injection. The values are means \pm S.E.

observed in the slow wave polyspike period. In some of them, though, I-CBF appeared to increase even further following cessation of EEG activity (e.g. thalamus, hippocampus, caudate putamen, pontine gray and cerebellar cortex). It is noteworthy that some structures (e.g. parietal, sensorimotor and auditory cortex, thalamus, inferior colliculus and cerebellar cortex) showed absolute flow rates exceeding 4 ml/g/min and that some (thalamus, hypothalamus, pontine gray and cerebellar cortex) had increases in flow to more than 300% of control. In a few structures (thalamus, hypothalamus and cerebellar cortex) individual animals showed excessively high flow rates with values grossly exceeding 5 ml/g/min.

The data of Table 2 and Fig. 1 were obtained by taking repeated densitometry readings from various parts of the same structure in consecutive sections (cf. Abdul-Rahman et al. 1979). In all groups except that studied after 15 min of ceased EEG activity the flow rates were fairly homogenous within each structure. However, in the 15 min group, I-CBF was inhomogenous in a few structures, particularly in parietal cortex. In these, I-CBF could vary from about 5 ml/g/min down to very low values.

Local CBF in the recovery period

The data given in Table 3 and Fig. 7 show that 5 min after glucose administration, hyperemia persisted

in some structures, particularly in thalamus, hippocampus and cerebellar cortex ($P < 0.01$). In structures of the cerebral cortex, though, I-CBF values had decreased to or slightly below control values. With prolongation of the recovery period, I-CBF decreased. In most structures, flow rates fell progressively in the period < 90 min. In some, I-CBF was reduced to or below 25% of control (auditory and visual cortex, hippocampus). In others, secondary underperfusion was either small (hypothalamus and colliculus inferior) or absent (pontine gray and cerebellar cortex). Prolongation of the recovery period to 180 min appeared to improve perfusion in some (but not all) structures. Thus, although the majority of the structures had flow rates that were grossly subnormal, some had I-CBF values lower than 30–35% of control.

Intracranial pressure

The pronounced reduction in local flow rates and the recovery period suggested that capillary circulation could have been impeded by increased intracranial pressure due to tissue edema. However, intracranial CSF pressure increased during the period of severe hypoglycemia (i.e. during the phase of hyperemia) and failed to increase during the recovery period (Fig. 3).

DISCUSSION

The present results confirm those obtained with different CBF techniques (Norberg & Siesjö 1970), showing that severe hypoglycemia is accompanied by a marked increase in CBF. Our results extend the previous ones to the local level and provide data for coma periods of up to 30 min. In addition, they yield information on I-CBF in the recovery period following normalization of blood glucose concentrations at the end of a 30 min period of ceased EEG activity. The results demonstrate that like conditions involving a restriction of oxygen supply (hypoxia and ischemia), substrate deficiency leads to vasodilatation in the brain. Moreover, like ischemia (Hossmann 1977), hypoglycemia is followed by a delayed hypoperfusion syndrome with local flow rates falling to very low values in the structures studied.

The results provide some interesting information on the mechanisms of the hypoglycemic hyperemia and the posthypoglycemic hypoperfusion. The hyperemia develops during the hypoglycemic

Intraglandular transport of ¹²⁵I-glandular kallikrein in the rat submandibular salivary gland

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ØRSTAVIK, T. B., GAUTVIK, K. M. & NUSTAD, K., Intraglandular transport of ¹²⁵I-glandular kallikrein in the rat submandibular salivary gland. *Acta Physiol Scand* 1980, 109: 315-323. Received 13 Nov 1979. ISSN 0001-6772. Department of Physiology and Biochemistry, Dental Faculty, University of Oslo, the Norwegian Radium Hospital, Oslo, and Department of Physiology, University of Oslo, Norway.

The transport of radiolabelled rat submandibular gland kallikrein was studied after local administration to the resting and activated rat submandibular gland. The iodinated kallikrein was electrophoretically, immunologically and biologically indistinguishable from the intact enzyme. After intraductal and intraglandular application the radioactivity in venous effluent was quantitated and characterized. As judged by gel-filtration ¹²⁵I-kallikrein in venous effluent eluted at a position similar to that seen when the iodinated enzyme was mixed with plasma, but earlier than the elution of ¹²⁵I-kallikrein in buffer. In plasma, therefore, glandular kallikrein is probably bound to macromolecules. The radioactive fractions in venous effluent did not contain free iodine. Maximum concentration of ¹²⁵I-kallikrein in venous effluent of resting glands was repeatedly reached about 20 min after intraductal administration. Moreover, the ductal epithelium represented the main permeation barrier since after intraglandular application the maximum venous ¹²⁵I-kallikrein concentration was reached almost immediately. In activated gland (parasympathetic and sympathetic nerve stimulation), the venous ¹²⁵I-kallikrein concentration was inversely related to glandular blood flow. We conclude that kallikrein present in the duct lumen or in the interstitium is able to reach the circulation, thereby making possible the local generation of plasma-kinins.

Key words: Glandular kallikrein, glandular transport

Glandular kallikreins (E.C.3.4.21.8) are present in many organs including the kidney and in the plasma from these organs (see Nustad et al. 1978). They are highly specific for their natural substrate kininogen and the biological function(s) of glandular kallikreins can be explained entirely or partly through the formation of lysyl-bradykinin and other kinins. Through the action of lysyl-bradykinin glandular kallikreins probably are involved in the regulation of blood flow in exocrine organs, and may also contribute to local permeability changes and the regulation of electrolyte transport (Nustad et al. 1978b).

Kallikreins are localized in the distal tubules of kidney (Ørstavik et al. 1976) and in the striated muscle of the salivary gland (Ørstavik et al. 1975; Indreberg et al. 1976; Ørstavik 1978; Højgaard et al.

1977). The enzyme is predominantly located in the apical part of the cells. This localization indicates a luminal secretory route, which is further substantiated by the presence of kallikreins in saliva and urine. The kallikrein-substrate kininogen, is present in plasma (Jacobsen 1966a) and has also been detected in the lymph (Jacobsen 1966b) and urine (Pisano et al. 1978). On the other hand, kallikrein has been detected in kidney perfusates (Roblero et al. 1976) and esterase activity possibly representing kallikreins has been detected in the renal lymph (deBono & Mills 1974). These findings indicate that at least some kallikreins may penetrate the tubule epithelium to interact with kininogen in the interstitial fluid. The subsequent formation of lysyl-bradykinin might cause vasodilatation and increased permeability of the blood vessels found in

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Intraglandular transport of 125 I-glandular kallikrein in the rat submandibular salivary gland

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The transport of radiolabelled rat submandibular gland kallikrein was studied after local administration to the resting and activated rat submandibular gland. The iodinated kallikrein was electrophoretically, immunologically and biologically indistinguishable from the intact enzyme. After intraoral and intraglandular application the radioactivity in venous effluent was quantitated and characterized. As judged by gel-filtration 125 I-kallikrein in venous effluent eluted at a position similar to that seen when the iodinated enzyme was mixed with plasma, but earlier than the elution of 125 I-kallikrein in buffer. In plasma, therefore, glandular kallikrein is probably bound to macromolecules. The radioactive fractions in venous effluent did not contain free iodine. Maximum concentration of 125 I-kallikrein in venous effluent of resting glands was repeatedly reached about 20 min after intraoral administration. Moreover, the ductal epithelium represented the main permeation barrier since after intraglandular application the maximum venous 125 I-kallikrein concentration was reached almost immediately. In activated gland (parasympathetic and sympathetic nerve stimulation), the venous 125 I-kallikrein concentration was inversely related to glandular blood flow. We conclude that kallikrein present in the duct lumen or in the interstitium is able to reach the circulation, thereby making possible the local generation of plasma-kinins.

Key words. Glandular kallikrein, glandular transport.

Glandular kallikreins (E.C.3.4.21.8) are present in many organs including the kidney and in the effluent from these organs (see Nustad et al. 1977). They are highly specific for their natural substrate, kininogen, and the biological function(s) of glandular kallikreins can be explained entirely or partly through the formation of lysyl-bradykinins and kininogens. Through the action of lysyl-bradykinin glandular kallikreins probably are important for the regulation of blood flow in exocrine glands, and may also contribute to local permeability changes and the regulation of electrolyte transport (Virstad et al. 1978b).

Kallikreins are localized in the distal tubules of the kidney (Ørstavik et al. 1976) and in the striated cells of the salivary gland (Ørstavik et al. 1975; Jakobsen et al. 1976; Ørstavik 1978; Høyem et al.

1977). The enzyme is predominantly located in the apical part of the cells. This localization indicates a luminal secretory route which is further substantiated by the presence of kallikreins in saliva and urine. The kallikrein-substrate kininogen is present in plasma (Jacobsen 1966a) and has also been detected in the lymph (Jacobsen 1966b) and urine (Prisano et al. 1978). On the other hand, kallikrein has been detected in kidney perfusates (Roblero et al. 1976) and esterase activity possibly representing kallikreins, has been detected in the renal lymph (deBono & Mills 1974). These findings indicate that at least some kallikreins may penetrate the tubule epithelium to interact with kininogen in the interstitial fluid. The subsequent formation of lysyl-bradykinins might cause vasodilatation and increased permeability of the blood vessels found in

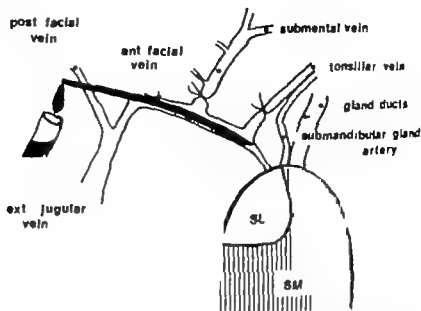


Fig. 1 Operating procedure. 125 I kallikrein was injected intraductally or intraglandularly into the submandibular gland. The venous effluent was collected by cannulation of the anterior facial vein after occlusion of the submental and tonsillar veins.

close proximity to the distal tubules (Beeuwkes & Bonventre 1975). In the salivary glands kallikrein activity has not been demonstrated in venous effluent except when artificial perfusates were used (Hilton 1970; Ørstavik et al. 1976). However release of kinins into the venous effluent of the dog submandibular gland has been observed following chorda tympani stimulation (Ferreira & Smaje 1976).

In order to examine the intraglandular transport of secreted kallikrein, purified rat submandibular kallikrein (Brandtzaeg et al. 1976) was labelled with 125 I and injected in microquantities subcapsularly or intraductally into the rat submandibular gland. Radioactivity associated with kallikrein was then measured in the venous effluent from the resting and activated gland. After application of 1 kallikrein intraductally or subcapsularly the labelled enzyme was recovered from blood draining the gland.

The initial part of this study has been reported elsewhere (Ørstavik et al. 1978).

MATERIALS AND METHODS

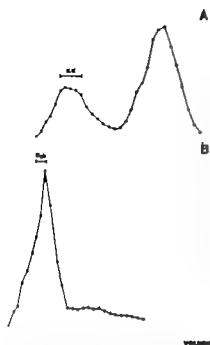
Animal and operative procedure. A total of 48 female and male Sprague-Dawley rats aged 3–4 months (250–350 g b.wt.) were used for the experiments. The animals were anesthetized with i.p. injections of pentobarbital (50 mg/kg b.wt.).

They were tracheotomized and had spontaneous breathing during the experimental period. All rats received heparin (0.06 ml 4000 I.E./ml) injected into the left femoral vein before cannulation of the blood vessel but after cannulation of the salivary duct.

Cannulation of the salivary duct. Cannulation of the submandibular gland main excretory duct as performed and described previously (Ørstavik & Clausen 1977). It was done prior to cannulation of any of the blood vessels. All glands were cannulated regardless of the approach route for the labelled kallikrein. The ability of the gland to respond to gland activation with secretion of saliva was controlled after the completion of the experiments. Saliva was collected in preweighed tubes and the volume was estimated by weight ($1 \mu\text{l} = 1 \text{ mg}$).

Procedure for blood sampling. Venous blood from the submandibular gland was obtained by cannulation of a vein from the submandibular and sublingual gland (Fig. 1). The cannula (Portex, PPS0) was inserted into the anterior facial vein proximal to the submandibular gland and pushed close to the branching point of the submandibular and sublingual veins. Prior to vein cannulation the tonsillar vein and the anterior facial vein distal to the tonsillar vein and the anterior facial vein distal to the submandibular gland branch were occluded. The cannula had to be separated from the submandibular gland artery and care was taken not to provoke bleeding or turbid glandular blood supply. Occlusion of the larynx was necessary to achieve reproducible results.

Blood was sampled into preweighed tubes and blood volume was estimated by weight ($1 \mu\text{l} = 1 \text{ mg}$). Blood was collected continuously and sampled at intervals of 10 min and blood flow rate thus recorded. Infusion was given i.v. through the right femoral vein during the experimental period. A Portex pump with a constant infusion rate was used and the infusion rate was



2 (A) Gel filtration of Sephadex G75 (20 \times 1 cm) of labelled kallikrein. Free radioactive iodine was eluted from the ^{125}I -kallikrein (KK). (B) Gel filtration of Sephadex G75 (11 \times 1 cm) of the effluent plasma (50 μl). The radioactivity eluted as one single peak close to the globulin-containing fractions (HB). The haemoglobin was easily detected by its red color. Thus, the radioactivity measured in the venous effluent did not represent alone, but eluted as a complex larger than kallikrein.

along in the effluent blood flow rate from the sublingual gland. Donor-blood was obtained by cardiac puncture of adult Sprague-Dawley rats, anesthetized and killed by heparin as described above. Parasympathetic activation was carried out as described previously (Járvai & Osvayik 1977). Parasympathetic activation obtained by electrical stimulation of the nerve plexus innervating the main excretory duct of the gland. Sympathetic stimulation was performed by subcutaneous electrical stimulation of the sympathetic cervical nerve chain. Plasma labelling of proteins. A highly purified rat sublingual gland kallikrein (Brändén et al. 1976) labelled using Na- ^{125}I -tyrosine and ^{125}I (Amersham). Labelled reaction mixture contained: 0.5 M Na-phosphate buffer pH 7.5 (50 μl), 0.5 M NaCl (5 μl), 24 μg Arg-His, 0.001 N NaClO in 0.05 M Na-phosphate buffer pH 7.5 (10 μl), and $\text{Na}_2\text{S}_2\text{O}_8$ (500 μl) of 1 g/l together with 10 μl KI and 1 g/l bovine serum albumin in 0.01 M phosphate buffer pH 7.5. The free and bound ^{125}I was separated on Sephadex G-75 columns. The final purified enzyme preparation had a specific activity of 4.5×10^5 cpm/ μg kallikrein assuming 75% recovery of protein. The iodinated kallikrein showed identical

Table 1 Concentration of ^{125}I -kallikrein in different tissues and fluids after intraductal microinjections in one representative experiment

| | cpm | % of radio-act. applied |
|--|-------------------|-------------------------|
| Applied ^{125}I -kallikrein (cpm/40 μl) | 3×10^6 | 100 |
| Glandular activity of ^{125}I -KK (cpm/g w w) | 2.0×10^6 | 38.9 |
| Maximal concentration in the venous effluent (cpm/ml) | 2.9×10^6 | |
| Total activity of ^{125}I KK in venous effluent collected during the first 22 min | 3.3×10^6 | 14.4 |
| Contralateral control submandibular gland (cpm/g w w) | 6.6×10^4 | 2.9 |
| Peripheral blood (cpm/ml) | 3 453 | |
| Thyroid glands (cpm/gland) | 1 064 | <0.1 |
| Saliva (parasymp) | | |
| 30 min after intraductal administration (cpm/min/11 μl) | 13 000 | 0.6 |
| 33 min after intraductal administration (cpm/min/5 μl) | 650 | <0.1 |

behaviour to the original kallikrein on the Sephadex G-75 column as radioimmunoassay (Nustad et al. 1978a), and enzymatically.

Human serum albumin and β -lactoglobulin was labelled with ^{125}I using Iodogen as an oxidizing agent (Fraker & Speck 1978). Free and bound ^{125}I was separated on P 100 columns. The final iodinated enzyme preparations had a specific activity of about 14×10^6 cpm/ μg assuming 75% recovery of the protein.

Measurement of rat submandibular gland kallikrein kinase activity. The hydrolysis of α -N-benzoyl-L-arginine ethyl ester (Bz Arg-OrEt) was used as a measure of kallikrein activity (Nustad et al. 1975).

Administration of iodinated kallikrein. Iodinated kallikrein (40 μl 1.3×10^6 cpm) was applied intraductally or intraglandularly to the submandibular gland. For the intraductal administration (Fig. 1), Hamilton syringe was connected to the polyethylene cannula which was fitted to the tip and inserted into the duct. The cannula was fastened with ligature and ^{125}I -kallikrein was infused (1–2 min) into the duct by slight manual pressure. When applied interstitially (intraglandularly), 4 aliquots of 10 μl were injected subcapsularly with Hamilton syringe.

Detection of kallikrein in venous blood and saliva. Appearance of ^{125}I -kallikrein in the blood was demonstrated by measurements of radioactivity in venous blood. The radioactive substance in blood was compared to the labelled kallikrein and to labelled kallikrein mixed with plasma *in vitro* by gel filtration on Sephadex-G75 columns. Total ^{125}I -kallikrein and ^{125}I -kallikrein concentrations in the blood samples were determined. Radioactivity in saliva

Table 2 Effects of intraductal microinjection on epithelial permeability

^{125}I - β -lactoglobulin and ^{125}I -albumin were injected intravenously in rats and radioactivity was assessed in saliva from both submandibular glands after activations with pilocarpine ($4 \mu\text{g/kg}$) given i.v. Prior to activation of the gland a intraductal sham injection was performed on one side. Saliva was collected in consecutive periods of 3 min, starting 7 min after the intraductal injection. A total of 27 collection periods from 11 different animals are given. Disruption of the epithelium caused by intraductal application was indicated in only 3 of these rats (+)

| Proteins | Dose $\times 10^6$ cpm | Collection periods | Radioactivity in saliva | | From contralateral control gland | | Radio- activity in blood (cpm/ml) |
|--|---------------------------|-----------------------|--|--------------------|-------------------------------------|--------------------|--|
| | | | After intraductal injection cpm/sample | cpm/ μl | cpm/sample | cpm/ μl | |
| ^{125}I - β -lactoglobulin (m.w. 35 000) | 40 | I | 714 | - | 723 | 12 | 85 |
| | | II | 228 | 9 | 260 | 12 | |
| | 40 | I | 2 232 | 13 | 300 | 16 | 75 |
| | | II | 900 | 9 | 889 | 10 | |
| | 40 | I | 1 574 | 1 | 1 575 | 12 | 66 |
| | | II | 579 | 7 | 482 | 8 | |
| | 4 | I | 171 | 1.6 | 139 | 2.2 | 9 |
| | | II | 71 | 1.2 | 73 | 2.6 | |
| | | III | 59 | 1.1 | 48 | 2.7 | |
| | | IV | 51 | 0.8 | 36 | 2.5 | |
| | 4 | I | 49 | 0.7 | 57 | 0.7 | 26 |
| | | II | 40 | - | - | - | |
| | | III | 35 | 1.2 | 38 | 1.0 | |
| | 4 | I | 165+ | 1.5+ | 45 | 1.1 | 28 |
| | | II | 77 | 1.0 | 72 | - | |
| | | III | 48 | 1.1 | 39 | - | |
| | | IV | 51 | 1.1 | - | - | |
| ^{125}I -albumin (m.w. 65 000) | 40 | I | 1 18 | 7 | 1 220 | 30 | 2 123 |
| | | II | 747 | 25 | 1 021 | 32 | |
| | 40 | I | 1 083+ | 26 | 611 | 26 | 2 122 |
| | | II | 793 | 44 | 708 | 59 | |
| | 40 | I | 761+ | 15 | 510 | 17 | 1 918 |
| | | II | 500 | 11 | 127 | 9 | |
| | 40 | I | 853 | 15 | 1 103 | 18 | 1 075 |
| | | II | 455 | 10 | 574 | 12 | |
| | 40 | I | 688 | 10 | 579 | 9 | 14 |
| | | II | 369 | 10 | 414 | 14 | |

and the gland was counted. For control purposes the radioactivity in the contralateral gland, in heart blood and in the thyroid glands were also recorded.

Control of disruption of ductal epithelium after intraductal application. The possibility that the intraductal injections had damaged the ductal epithelium was tested by studying the transport of proteins from blood to saliva. The main duct of both submandibular glands was cannulated. A Hamilton syringe was connected to one of the two glands for intraductal application of buffer as described above. After the intraductal application ^{125}I labelled reference proteins were administered through the femoral vein. 7 min after the intraductal injection pilocarpine ($0.4 \text{ ml } 0.4\%$) was administered i.v. and saliva collected from both glands in consecutive periods of 3 min each.

Radioactivity was measured in the salivary sample, in blood and in the thyroid and the submandibular glands. Kallikrein apparently combines with compounds in plasma so that its molecular weight and then its transport properties may be altered (Nustad et al. 1978a). Transport of ^{125}I -kallikrein from blood to saliva may therefore be comparable to the transport from duct lumen to blood, since no binding-substances exist in saliva. Instead β -lactoglobulin (m.w. 35 000) and human serum albumin (m.w. 65 000) rather than kallikrein were chosen for these control experiments. These proteins were labelled with ^{125}I as described above and given in dose equaling $1.1 \mu\text{g}$ protein (40×40^6 cpm) and also $0.1 \mu\text{g}$ protein (4×10^6 cpm) for β -lactoglobulin.

Histological examination

Results The effect of

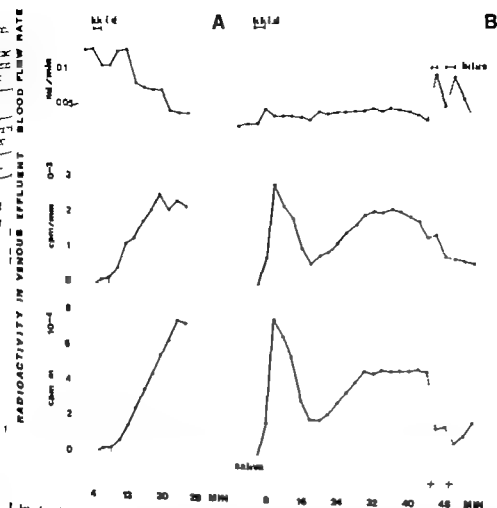


Fig. 1. Intraductal administration of 125 I-kallikrein into the resting submandibular gland of two different rats (A and B). The effect of the injection procedure is shown as an initial peak in the concentration of radioactivity in venous effluent (cpm/min) (B). In spite of this, the gland responded in the regular fashion to parasympathetic stimulation (saline) at the end of the experimental period with increased blood flow. At rest, maximum concentration of 125 I-kallikrein in venous blood is repeatedly reached about 20 min after the initial application.

Procedures for 125 I-kallikrein application on gland physiology is controlled after injection of phosphate buffered saline (PBS, 0.01 M Na-phosphate buffer pH 7.4, 0.15 M NaCl) intraductally or subcutaneously. The glands are excised, fixed in 96% ethanol, brought through 1-ethanol, and embedded in paraffin. Tissue sections (6 μ m) were stained to demonstrate endogenous kallikrein activity (Ortengren et al. 1975). The tissue sections were especially examined for the presence of endogenous kallikrein activity as an indication of disruption of gland cells.

Drugs: Pentobarbital (Nembutal sodium[®] Abbott Laboratories, London), heparin (Heparin Leo 5000 J[®] Lævring Kemiisk Fabrik, Ballerup, Denmark),

phloretin (NAF-Laboratories A/S Oslo, Norway), Bovine serum albumin[®], β -lactoglobulin[®] and α -n-benzoyl-L-arginine ethyl ester HCL[®] (Sigma, St. Louis, MS, USA), Human serum albumin[®] (ORHA 0405 Behringwerke AG Marburg, W. Germany), Sephadex G-75[®] (Pharmacia Fine Chemicals Uppsala, Sweden), P 100 (BioRad Laboratories, Richmond, California).

RESULTS

Characterization of 125 I labelled kallikrein. The iodinated enzyme was homogenous as judged by gel-filtration on Sephadex G-75 (Fig. 2 A). The 125 I-

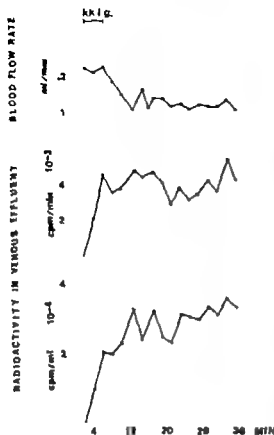


Fig. 4 Intraglandular (interstitial) application of 125 I-kallikrein in the resting submandibular gland. The concentration of 125 I-kallikrein in venous blood (cpm/ml) increased rapidly, reaching a plateau after about 10 min.

kallikrein behaved immunologically (Nustad et al 1978a) as the intact enzyme. Moreover the iodination procedure did not alter the enzymatic activity of kallikrein, since the Bz-Arg-OMe-esterase activity of kallikrein (204–702 EU/mg protein) was not different from that of kallikrein labelled with 125 I (208–202 EU/mg protein). The radioactivity in the gland venous effluent after intraductal and subcapsular application was excluded when filtered on Sephadex G-75 (Fig. 2B). This is in agreement with the binding of kallikrein to inhibitor-proteins in plasma as seen when 125 I-kallikrein was mixed with plasma *in vitro* (Nustad et al 1978a). There was only a very small release of free 125 I from labelled kallikrein since the radioactivity of the thyroid glands was negligible after the experiments (Table 1) and no free iodine was recovered in the venous effluent after gel-filtration (Fig. 2B). Therefore measurement of radioactivity in the venous effluent after injection of 125 I-kallikrein is a useful

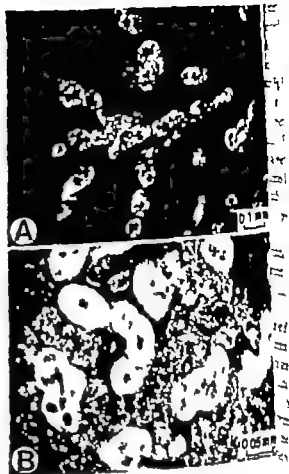


Fig. 5 Kallikrein-specific immunofluorescence in submandibular gland duct cells. When 125 I-kallikrein administration did not permanently interrupt ductal cell integrity (A), the 125 I-kallikrein concentration curve resembled that shown in Fig. 3. However an injection which interrupted the ductal cell morphology resulted in a rapid diffusion of endogenous kallikrein (B). In these glands 125 I-kallikrein concentration curve resembled that seen after an intraglandular administration as shown in Fig. 4.

marker for the intraglandular distribution of kallikrein.

Tissue effects of the injection procedure. The possibility that the intraductal injections had disrupted the epithelial barrier and thereby given rise to misconceptions was tested in several ways. 1. Disruption of the epithelial permeation barrier was recognized by an immediate and high increase in the concentration of venous effluent radioactivity. The degree of tissue trauma was indicated by the size of this peak, which varied from being absent (Fig. 3A) to a pattern resembling that of an intraglandular administration (Fig. 4). Most often a transient initial peak was detected (Fig. 3B). Complete absence of this peak was only seen (Fig. 3C).

morphologically recognizable disruption of ducts and diffusion of endogenous kallikrein were never observed in tissue sections (Fig. 5A) permeants with transient initial rise in ^{125}I -kallikrein concentration (Fig. 3B). However when intraductal application of ^{125}I kallikrein produced a permeation curve resembling that of an intravascular administration (Fig. 4) disruption of ductal integrity was observed as indicated by gross leakage of endogenous kallikrein (Fig. 5B). Also when morphologically recognizable changes of the epithelium were present, the glands often reacted in a normal way to activation (Fig. 3B) but the intraductal application did not usually cause a permanent damage of the ductal wall. This was demonstrated by studying the transport of labelled β -lactoglobulin and albumin from blood to gland. In eight out of eleven rats the total amount of the concentration of iodinated protein found in the gland was the same as in the gland which had received an intraductal injection as in the contralateral control gland (Table 2).

These experiments show that the ductal integrity of these glands was usually not permanently damaged by the intraductal application procedure. In these experiments it was concluded that the permeation curve of the ^{125}I -kallikrein concentration curve is the best indication of the extent of disruption of the duct epithelial barrier after the intraductal administration. Results in electrically stimulated glands are disregarded when this concentration curve indicated a persistent leakage of the ductal barrier.

Transport of ^{125}I -kallikrein in resting glands. Intravascular microinjection of ^{125}I -kallikrein resulted in a rapid increase in the specific radioactivity (cpm/ml) in glandular blood. Maximum concentration was observed after about 20 min and it remained high for some time before declining (Fig. 3B). In contrast, after intraglandular injections of ^{125}I -kallikrein, a maximum blood concentration was achieved almost immediately (Fig. 4). The results show that ^{125}I -kallikrein is able to penetrate the duct epithelium and enter the blood stream. The duct epithelium seemed to represent the main permeation barrier. Since intravascularly applied ^{125}I -kallikrein was readily transported into the circulation. The application of ^{125}I kallikrein both intraductally and subcapsularly induced an increase in glandular blood flow (Fig. 3). This increase could be duplicated by saline

(data not shown) and was therefore not due to the minute amounts of injected exogenous kallikrein.

Diffusion of ^{125}I -kallikrein in activated glands. Parasympathetic nerve stimulation of the gland resulted in saliva formation and increased glandular blood flow (Fig. 3B). Concomitantly there was a parallel increase in total blood radioactivity but a decreased concentration of ^{125}I -kallikrein in venous blood cpm/ml (Fig. 3B). The effect of the parasympathetic stimulation was the same whether the ^{125}I -kallikrein was administered intraglandularly or intraductally. Glandular effluent blood flow and the concentration of venous ^{125}I -kallikrein after parasympathetic nerve stimulation were inversely related (correlation factor was -0.70). Stimulation of the sympathetic nerve induced secretion of saliva and a vasoconstriction which was followed by a vasodilatation. After both intraductal and intraglandular administration of ^{125}I -kallikrein the stimulation was followed by a parallel increase in blood flow and total venous radioactivity whereas the ^{125}I kallikrein concentration was inversely related to glandular blood flow.

DISCUSSION

The purpose of the present study was to examine the intraglandular transport of submandibular gland kallikrein after intraductal and interstitial application. The rat submandibular gland was used since autologous purified glandular kallikrein (Brandtzaeg et al 1976) was available. The radioactively labelled kallikrein was immunologically chromatographically and biologically identical to the untreated enzyme. When introduced artificially into the duct lumen, the ^{125}I -kallikrein could be used to study the transport of kallikrein within the gland in situ. With the intraglandular application of ^{125}I kallikrein, precaution was taken not to introduce permeability artifacts due to increased intraluminal and interstitial pressure. Impairment of the ductal epithelial barrier by intraductal administration of kallikrein was best examined by following the appearance of ^{125}I kallikrein in venous blood. An immediately occurring peak of varying size in the ^{125}I kallikrein concentration in the venous effluent was believed to represent penetration across the epithelium caused by increased intraductal pressure. When this effect was pronounced duct epithelial cells were destroyed and could be detected immunohistochemically by gross diffusion of

endogenous kallikrein from the ductal cells. The radioactivity concentration curve then resembled that seen after interstitial application. In morphologically unaltered glands the transient increase in ¹²⁵I kallikrein concentration was followed by a second rise which was comparable in time course and magnitude to that seen when the initial peak was absent. This late increase in ¹²⁵I kallikrein concentration in venous blood which reached its maximum about 70 min after the intraductal injection was believed to reflect the transport of endogenous enzyme across the epithelial barrier.

Transport of iodinated proteins from blood to saliva was studied in paired glands 7 min after one of these glands had been subjected to an intraductal sham injection. Increased permeation was observed in only 3 out of 11 animals. These results further indicate that the ductal epithelium is not usually damaged by an intraductal application. In these experiments albumin and β lactoglobulin were used as tracer proteins. The much higher ratio between saliva and blood concentration of β -lactoglobulin as compared to albumin probably reflects the difference in molecular weight i.e. 35 000 and 65 000 respectively since molecules with a molecular weight above 45 000 will be retained also by the basal membrane (Porter & Bonneville 1968). The amount of radioactivity in the submandibular glands did not differ between β lactoglobulin and albumin (data not shown). Thus it does not seem likely that the difference in saliva to blood concentration ratio is due to an accumulation of β lactoglobulin in the submandibular gland.

Permeation of radiolabelled kallikrein across an epithelial barrier has previously been shown in the intestine (Moriwaki et al 1973) and salivary kallikrein has been found to penetrate the oral gingival epithelium in an active form causing vasodilatation and periodontal inflammation (Barabash et al 1976). Moreover, esterase activity possibly representing kallikrein has been detected in the renal lymph (de Bono & Mills 1974) and kallikrein has been found in kidney perfusate and urine of isolated rat kidneys (Roblero et al 1976). The present study shows that kallikrein may also penetrate the ductal epithelium of the rat submandibular gland. The ductal epithelium evidently represents the main permeation barrier.

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endogenous kallikrein from the ductal cells. The radioactivity concentration curve then resembled that seen after interstitial application. In morphologically unaltered glands the transient increase in ^{125}I kallikrein concentration was followed by a second rise which was comparable in time course and magnitude to that seen when the initial peak was absent. This late increase in ^{125}I kallikrein concentration in venous blood which reached its maximum about 20 min after the intraductal injection was believed to reflect the transport of endogenous enzyme across the epithelial barrier.

Transport of iodinated proteins from blood to saliva was studied in paired glands 7 min after one of these glands had been subjected to an intraductal sham injection. Increased permeation was observed in only 3 out of 11 animals. These results further indicate that the ductal epithelium is not usually damaged by an intraductal application. In these experiments albumin and β -lactoglobulin were used as tracer proteins. The much higher ratio between saliva and blood concentration of β -lactoglobulin as compared to albumin probably reflects the difference in molecular weight, i.e. 35 000 and 65 000 respectively, since molecules with a molecular weight above 45 000 will be retained also by the basal membrane (Porter & Bonneville 1968). The amount of radioactivity in the submandibular glands did not differ between β -lactoglobulin and albumin (data not shown). Thus it does not seem likely that the difference in saliva to blood concentration ratio is due to an accumulation of β -lactoglobulin in the submandibular gland.

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nonary O₂ transfer during pulsatile non-pulsatile perfusion

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HAUGE, A. & NICOLAYSEN, G. Pulmonary O₂ transfer during pulsatile and non-pulsatile perfusion. *Acta Physiol Scand* 1980, 109: 325-332. Received 20 Nov. 1979. ISSN 0001-6772. Institute of Physiology, University of Oslo, Norway.

The importance of the perfusion pattern for the oxygen transfer has been examined in isolated rabbit lungs perfused with plasma at constant volume inflow. The lungs were ventilated with constant tidal volume and constant end-expiratory pressure. Following a standardized rise in P_{iO_2} , the rate of rise in pulmonary venous P ($\Delta P_v/\Delta t$) was measured during alternately pulsatile and non-pulsatile perfusion in normal lungs and in lungs made edematous by elevation of left atrial pressure. In normal lungs there was no difference in $\Delta P_v/\Delta t$ when the two modes of perfusion were compared. In edematous lungs $\Delta P_v/\Delta t$ was statistically higher during pulsatile perfusion, indicating a beneficial effect of flow and pressure pulsations, e.g. a better distribution of V/Q ratios throughout the lungs. In a separate series of experiments the advancement of high O₂ front through the airways was measured, and the two perfusion patterns compared. Since no difference was found, we suggest that the phenomenon of cardiogenic gas mixing in the airways as such is result of direct action of the heart on the lungs rather than arterial pulsations.

Key words: Isolated lungs, gas mixing, respiration, lung edema, gas exchange diffusion

Theoretical effect of pulmonary blood flow velocity on gas exchange have been calculated and discussed by several workers, e.g. Crandall & Wierick (1967), Flumenfeldt & Crandall (1968), Wierick et al. (1969), Lin & Cunniff (1973) and Wierick & West (1972), all of whom found that flow velocity was detrimental to gas exchange. Recent work by Bidani et al. (1978) pointed out that varying parameters in a physico-chemical system cause pulsatile capillary flow and volume result in improved performance when compared to a situation when only one parameter is non-steady ("parametric pumping"). They developed a mathematical model which allowed for such effects to occur using the pulse contours measured by Wierick et al. (1970). Their results suggest that alternating pulsatile pulmonary capillary blood flow and volume can lead to an oxygen exchange as efficient as in the case of constant flow and volume and is more efficient than either pulsatile flow or pulsatile volume alone.

One turn from the many theoretical treatises to

examine the experimental evidence one will find few works which directly compare alveolar-capillary gas exchange during pulsatile and non-pulsatile flow conditions. Gillespie et al. (1970) perfused one dog lung with pulsatile or non-pulsatile blood flow and measured the rate of uptake of CO and ¹⁵O₂. They found that pulsatile flow increased the uptake of both gases, CO more than ¹⁵O₂. Based on studies of pulsatile vs. non-pulsatile dog lung perfusion Clarke et al. (1968) on the other hand suggested that O₂ uptake did not change between the two conditions. Their data on gas exchange were rather scarce, however.

In the present work we have monitored venous (effluent) P after standardized rise in P_{iO_2} during alternating pulsatile and non-pulsatile perfusion of rabbit lungs. Hemoglobin-induced facilitation of diffusion and other chemical effects due to hemoglobin were excluded from consideration by using plasma as perfusate, focusing the attention on pure physical effects of flow pulsatility. Our aim was to provide experimental data which could be of help

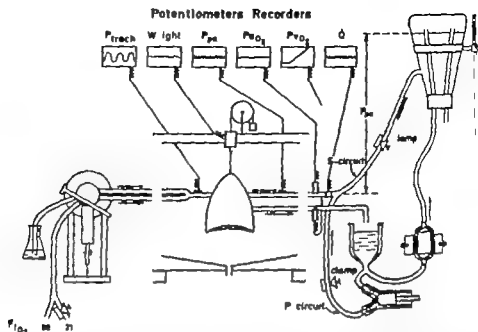


Fig. 1 The experimental arrangement for perfusion of isolated rabbit lungs with alternately pulsatile (P-circuit) and steady (S-circuit) flow. A detailed description is given in the text. P_{trach} = tracheal pressure, P_{pa} = pulmonary arterial pressure, P_{po_2} = pulmonary arterial P , P_{vd_2} = pulmonary venous P , Q = flow, F_{10_2} = fraction (vol/vol) of oxygen in dry ventilation gas mixture.

when the relative merits of the many theoretical studies in this field are assessed.

METHODS

Isolated perfused lungs from albino rabbits of either sex weighing 3.0–4.0 kg were used. The lung donor animal was anesthetized with pentobarbital (Nembutal, Abbott) 30–50 mg/kg i.v. The procedure for removal of the heart and lungs was as described by Hauge et al. (1966). The chest was opened under positive pressure ventilation which was maintained until the circulation was stopped. Just prior to the cardiac arrest, heparin (750 IU/kg) was given i.v. Perspex cannulas were placed in the left auricle and via the right heart ventricle into the pulmonary artery. The perfusion and ventilation arrangement is demonstrated in Fig. 1.

The lungs were suspended underneath a force transducer (Sanborn FTA 100-1) connected to the heart and the cannulas inside a thermostated (38°C) Perspex chamber. Lung weight was continuously monitored. The lungs were ventilated with constant stroke volume, positive pressure and with a stroke frequency from 34 to 38/min. The ventilator was a Starling 'Ideal' ventilation pump (shown schematically to the left in Fig. 1). End-expiratory pressure was set with a waterseal as demonstrated and inflation pressure (P_{wght}) was recorded by connecting a pressure transducer (Hewlett Packard Model 70) to a side branch of the tracheal cannula. Equidistant from the inlet of the ventilation pump were positioned two gas-filled balloons, one containing air with 4% CO_2 , the other 96% O_2 and 4% CO . At a standardized point of time in the ventilation

cycle one could by this arrangement, switch from ventilation with normal air to ventilation with high oxygen load (F_{10_2}). The effect of a change in F_{10_2} on the partial pressure of oxygen in the effluent plasma (P_{po_2}) is recorded with a thermostated Radiometer O_2 electrode connected to a Beckman Model 160 oxygen meter. Membrane thickness was 5 to 6 μm .

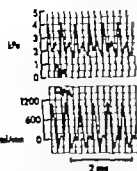
Pulmonary arterial pressure (P_{pa}) was measured by catheter from a sidearm of the arterial cannula (Sofa transducer P23De).

At the start of perfusion left atrial pressure (left pressure) was kept at 0–0.1 kPa. In the experiments involving hydrostatic pulmonary edema, left atrial pressure elevation was carried out by leading the perfusate coming from the left atrium through the desired elevation in a vertical ladder of glass and rubber tubing (included in Fig. 1) the pressure being either 1.3 or 1.6 kPa. Zero reference for both in- and outflow pressures was the level of the openings of the perfusion cannulas, 5 cm below the top of the lungs. The total height of the left was about 8 cm.

From the thermostated venous reservoir the perfusate could follow one of two alternative routes into the pulmonary artery as shown in Fig. 1.

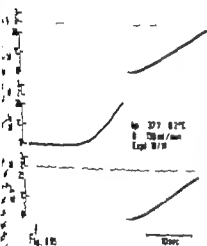
The P-circuit. The perfusate could be pumped from the reservoir directly into the pulmonary arterial tubing (Harvard pulsatile blood pump Model 147). The flow volume and the frequency was adjusted to give a flow within the normal range.

The S-circuit. Alternatively the perfusate could be pumped with a variable speed peristaltic pump (Harvard Model 1210) to a second thermostated venous reservoir from which it flowed passively into the pulmonary artery.



Typical examples of pulmonary arterial pressure (P_{pa}) recordings during pulsatile perfusion.

h. The height of this second reservoir above the of the openings of the perfusion cannulas could be of to give any desired pulmonary arterial pressure. experiment was always started using pulsatile flow gives typical example of the pulmonary arterial and flow contours. In order to avoid vasoconstriction variations in vascular resistance 13 mg trane chloride was added to the perfusate. By altering between the two perfusion circuits, maintaining mean flow, the effect of switching from low to F_{low} could be studied repeatedly during the



Three recordings of changes in pulmonary venous (P_{pv}) following a step rise in F_{low} (at arrow) from 0.1 to 0.95. Responses obtained during steady (non-pulsatile) flow are shown in the top and the bottom sections. Response obtained during pulsatile flow is shown in the middle section. Mean flow was kept constant throughout experiment. The figure demonstrates the differences in the of the P_{pv} curve ($\Delta P_{pv}/\Delta t$) and the differences in time elapsed from the start of high oxygen ventilation (P_{pv} begins to rise ("time to take-off"). Also shown is constant pulmonary arterial pressure (P_{pa}) recording. During the first part of the pulsatile flow period the pressure recording was electrically damped.

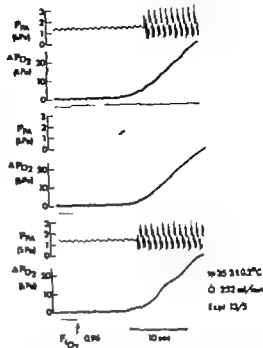


Fig. 4. Three recordings of changes in pulmonary end-tidal PO_2 (ΔP) following a step rise in F_{low} (at arrow) from 0.1 to 0.95. Responses obtained during pulsatile flow are shown in the top and bottom sections. A response obtained during steady (non-pulsatile) flow is shown in the middle section. Mean flow was kept constant. Also shown are the constant pulmonary arterial pressure (P_{pa}) recordings. During the first part of the pulsatile flow period the pressure recordings were electrically damped.

to modes of perfusion. Flow was measured by flow-through transducer connected to a Nycotron 376 square-wave electromagnetic flowmeter. Pressure, weight, flow, and P_{pa} signals were recorded on Hewlett Packard 7402A Oscillograph recorder and on Sanborn Model 320 dual-channel amplifier recorder.

Hanger & Nicolayson (1971) found that the lung preparation used in the present studies had normal ultrastructure without interstitial edema after several hours of perfusion with pulsatile plasma flow at physiological vascular pressures and normal vascular resistance.

In one experiment, the transit of Evans blue through the lungs during pulsatile and non-pulsatile flow was examined. The experimental setup was identical to that described above, except that the total venous outflow from the preparation could be diverted to a rapid sampling system (Nicolayson 1971). By this system the outflow from the lungs could be sampled in split fractions with one sample per 0.67 s. Since the total outflow could be sampled there was no recirculation. Initially pulsatile flow was established. During this perfusion 0.3 ml of solution of Evans blue in horse plasma was rapidly injected into the tubing to the pulmonary artery. The injection was performed in this and the subsequent tests at peak inspiration. Simultaneously

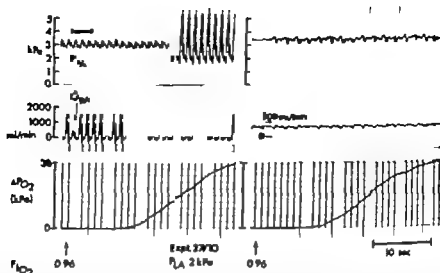


Fig. 5 Two recordings of changes in pulmonary venous P (ΔP) following step rises in F (at arrows) from 0.1 to 0.96 in a situation of ongoing transvascular fluid filtration. Also shown is the concomitant pulmonary arterial pressure (P_A) and flow recordings (Q). Mean flow was kept constant.

the sampling of the outflowing plasma was started. The perfusion was then changed to nonpulsatile and injections of Evan blue and sampling of the venous outflow performed. Then pulsatile flow was reestablished and a 4th injection/sampling done. The samples of the outflow were weighed and the optical density (O.D.) at 600 nm determined.

In one group of experiments designed to study airway diffusion the lungs were not ventilated. Transpulmonary pressure was maintained constant by connecting the trachea to a pressurized gas tank via a gas flowmeter and an overflow system. The experiment was done by alternating between air (with 4% CO_2) and oxygen- (with 4% CO_2) containing gas tank. The tubings and gas flow connections were designed in such a way that gas switching could take place without lung movement. In this particular series of experiments the content of oxygen in the tracheal gas was measured continuously via a small bore catheter by a Centronic mass spectrometer 700 MGA connected to a Rikadenki recorder.

RESULTS

The kinetics of airway to blood oxygen transfer during alternately pulsatile and non pulsatile perfusion was assessed by two variables:

(1) $\Delta P_{O_2}/\Delta t$ which is the slope of the middle 1/3 of the recorded P_{O_2} range and

(2) Time to "take-off" which is the time from switching to high oxygen (F_i 0.96) ventilation gas to the point when venous oxygen pressure (P_{O_2}) started to rise.

The two variables are shown in Fig. 3 which demonstrates a particularly large difference be-

tween pulsatile and non pulsatile flow both in time to "take-off" and $\Delta P_{O_2}/\Delta t$ are considered though the results demonstrated in this review are atypical the figure is included since it clearly demonstrates what was measured.

The P_{O_2} obtained during ventilation with 4% CO_2 was set on the recorder baseline. recorded P range above this value was on average 24 kPa (182 mmHg). The shape of the curve above this range was not considered. Fig. 4a demonstrate typical results obtained in normal edematous lungs respectively. Fig. 5 includes recordings (Q_{PA}) in addition to pulmonary arterial pressure (P_A) recordings. The relatively high pulmonary arterial pressure in the latter example is to the elevated left atrial pressure (2 kPa) necessary to induce a hydrostatic type of lung edema.

An interesting by-observation was made during the venous oxygen pressure. The oscillations occur both during pulsatile and nonpulsatile per-

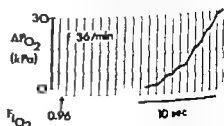


Fig. 6 A recording of pulmonary venous P_{O_2} following a step rise in F from 0.1 to 0.96 during pulsatile perfusion (f = ventilation frequency).

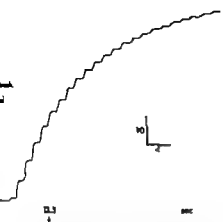


Fig. 7 The change in tracheal P_{O_2} measured by mass spectrometry following ventilation with gas mixture containing 9% per cent O_2 began at time zero (first arrow). 17.5 min shows P_{O_2} as measured with an O_2 electrode 4 cm to the (second arrow). Tidal volume was 76 ml and capacity 175 ml. The relatively slow build-up of the tracheal P is to be noted due to the size of the tidal volume relative to anatomical dead space (up to the gas sampling point).

In most cases with the same frequency as the balloon, i.e. from 3 to 36 per min. See Fig. 4 for tracing and Fig. 6. Such oscillations were related to the size of the tidal volume relative to anatomical deadspace of the lungs and the ventilation rate, as well as the dimensions and internal condition of the connections from the gas balloon to the tracheal cannula. Fig. 7 gives an example of the time pattern of increase in tracheal P following a rise in F_{IO_2} . Tracheal air was sampled continuously from the level of the bronchial bifurcation and analyzed with mass-spectrometer. The tidal volume, the ventilation frequency, the space and the gas-connections were similar to those used during the experiments listed in Tables 1 and 2.

Table 1 gives an overview of the results obtained in non-edematous lungs. Mean flow was kept constant in each perfusion experiment. There was no systematic effect on pulmonary vascular resistance (PVR) of going from pulsatile to non-pulsatile flow and vice versa. Two pairs of lungs (expts. 11/11 and 17/1) had relatively high PVR, flow 138 and 120 ml/min, respectively. Mean flow for the other 14 lungs was 56 ml/min, and the average pulse frequency 60 per min. Mean P in all the lungs listed in Table 1 was 1.46 kPa, average pulse

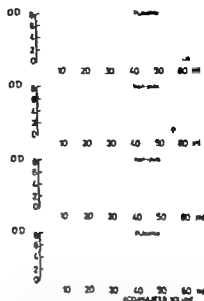


Fig. 8 Evans blue concentration in pulmonary outflow following bolus injection in the pulmonary artery during pulsatile and non-pulsatile perfusion. The bolus injections were performed at zero accumulated volume. The plasma flow in the four tests was from above: 403, 294, 394 and 331 ml/min.

pressure 1.98 kPa, peak inflation pressure 0.48 kPa, and end-expiratory pressure was set at 0.1 kPa.

$\Delta P/\Delta t$ was not significantly different when the two modes of perfusion were compared ($P=0.18$, Sign-test, two-sided). Time to take-off was shorter in lungs perfused with pulsatile flow ($P=0.004$).

Table 2 gives the results from lungs with hydrostatic lung edema. Left atrial pressure was elevated to either 1.3 or 2.0 kPa, and extravascular fluid accumulation was monitored gravimetrically. On the average 9.4 g of extra fluid can accumulate in the interstitial space before alveolar flooding occurs (Hauge et al. 1975). The fluid accumulation values listed in the table are those at the end of the experiment.

We found without exception that $\Delta P_{O_2}/\Delta t$ was higher during pulsatile than during non-pulsatile perfusion, i.e. the slope of the P_{O_2} was steeper. Furthermore we found that in edematous lungs time to "take-off" was always shorter during pulsatile than during non-pulsatile perfusion.

The difference in the time to "take-off" which was observed in both normal and edematous lungs could be due to a shorter blood transit time in the vascular bed, a higher mean linear gas velocity in the airways during pulsatile flow or both. We de-

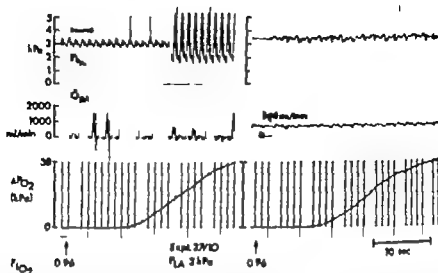


Fig. 5. Two recordings of changes in pulmonary venous P (ΔP_{v}) following step rises in I (at arrows) from 0.1 to 0.96 in a situation of ongoing transvascular fluid filtration. Also shown is the concomitant pulmonary arterial pressure (P) and flow recordings (Q). Mean flow was kept constant.

the sampling of the outflowing plasma was started. The perfusion was then changed to non-pulsatile and injections of Evans blue and sampling of the venous outflow performed. Then pulsatile flow was re-established and a 4th injection/sampling done. The samples of the outflow were weighed and the optical density (O.D.) at 600 nm determined.

In one group of experiments designed to study airway diffusion the lungs were not ventilated. Trans pulmonary pressure was maintained constant by connecting the trachea to a pressurized gas tank via a gas flowmeter and an overflow system. The experiments were done by alternating between air (with 4% CO_2) and oxygen (with 4% CO_2) containing gas tank. The tubings and gas flow connections were designed in such a way that gas switching could take place without lung movement. In this particular series of experiments the content of oxygen in the tracheal gas was monitored continuously via a small bore catheter (Lyn Centricon mini spectrometer 700 MGA connected to a Rikadenki recorder).

RESULTS

The kinetics of airway to blood oxygen transfer during alternately pulsatile and non-pulsatile perfusion was assessed by two variables:

- (1) $\Delta I / \Delta t$ which is the slope of the middle 1/3 of the recorded P range and
- (2) Time to 'take-off' which is the time from switching to high oxygen ($I = 0.96$) ventilation gas to the point when venous oxygen pressure (P) started to rise.

The two variables are shown in Fig. 3 which demonstrates a particularly large difference be-

tween pulsatile and non-pulsatile flow both time to 'take-off' and $\Delta P / \Delta t$ are considered though the results demonstrated in this report are atypical the figure is included since it will clearly demonstrate what was measured.

The I_{CO_2} obtained during ventilation with 4% CO_2 was set on the recorder baseline recorded I range above this value was an average 24 kPa (180 mmHg). The range above this range was not considered (Fig. 4 demonstrate typical results obtained in non-edematous lungs respectively Fig. 5 include recordings (Q) in addition to pulmonary pressure (P) recordings. The relatively high pulmonary arterial pressure in the latter example to the elevated left atrial pressure (2 kPa) seemed to induce a hydrostatic type of lung edema.

An interesting by-observation was oscillation of the venous oxygen pressure. The oscillation occurs both during pulsatile and non-pulsatile

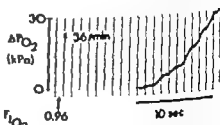


Fig. 6. A recording of pulmonary venous P following a step rise in I from 0.1 to 0.96 pulsatile perfusion (f = ventilation frequency).

measured the appearance and the concentration versus outflow volume of E and blue in one perfused preparation (Fig. 8). The results indicate that the appearance of the tracer and the peak concentrations are found in the same accumulated outflow volume and occurs outflow after the time of injection. Since the flow was not exactly the same during the 4 tests, accumulated volume was used instead of time. The range of the outflow volume was the same during pulsatile and non-pulsatile perfusion.

Finally we compared the advancement of the oxygen front in inflated but unventilated lungs during pulsatile and non-pulsatile perfusion. At a certain point of time the positive pressure inflation source was changed from air (with 4% CO₂) to gas (with 4% CO₂) and the time which elapsed until arterial P_aO₂ and venous P_vO₂ started to increase, was recorded. The results are listed in Table 1. There was no systematic difference when the modes of perfusion were compared under conditions of equal outflow.

DISCUSSION

The most important observation made during the present series of experiments was that the gas-exchange in normal (non-edematous) lungs, measured as $\Delta P_a/\Delta t$ was equally efficient during pulsatile and non-pulsatile flow. The investigation does not support to the many theoretical studies which conclude that flow pulsatility is detrimental to gas exchange. The result is compatible with the introduced by Bidani et al. (1978), outlined in edematous lungs pulsatile blood flow was more efficient than a non-pulsatile mode of flow. Several workers have observed that interstitial edema tends to cause flow deviations away from the edematous regions (West et al. 1965; Ghes et al. 1968; Diff et al. 1972). The relative increase in vascular transmural pressure swings during pulsatile flow may well contribute to maintain the efficiency of the lung vessels in the presence of edema and in this way preserve a more normal alveolar ventilation/perfusion relationship. Diff et al. (1972) found that interstitial edema tended to cause a decrease in terminal airway resistance and even terminal airway closure. The deviations in flow and ventilation were not matched, however. If the terminal

airway dimensions are better maintained during pulsatile flow the build-up of alveolar P following a rise in F₁₀ would be more rapid. This would be an alternative or a complementary explanation of the present findings.

In our opinion the measured variable 'time to take-off' is less informative and more related to the particulars of the experimental design than $\Delta P/\Delta t$. Clearly the long absolute time which elapsed from the low to the high F₁₀ switch until venous P started to rise to a large extent reflected the distances and the dimensions of the external gas connections. A main determinant for 'time to take-off' was therefore the build-up of a higher alveolar P_a. This does not however explain the statistical difference between pulsatile and non-pulsatile perfusion since the geometry of the experimental set-up was the same. Furthermore the difference was present regardless of the occurrence of lung edema.

Two sets of experiments were carried out with the purpose of finding an explanation. We reasoned that the transit time for plasma from the alveolus to the position of the oxygen electrode could be shorter during pulsatile than during non-pulsatile perfusion and/or that the mean linear gas velocity in the airways could be higher due to arteriogenic gas mixing. The results from indicator dilution experiments (Fig. 8) made the first of these two hypotheses less probable so we turned our interest to the gas movement in the airways.

During a normal inspiration gas moves through the pulmonary airway by a combination of convection and diffusion. In the terminal airways and the alveolar ducts molecular diffusivity of oxygen is assumed to be sufficiently great in relation to local dimensions to completely mix the inspired with alveolar gas (Fukuchi et al. 1976). In the larger airways however where linear dimensions are greater molecular diffusion is insufficient, resulting in a concentration gradient or front between inspired and alveolar gas. Several studies have demonstrated that the mechanical action of the heart enhances gas mixing in the lung, both during breath holding (Engel et al. 1973) and during inspiration (Fukuchi et al. 1976). This effect has been called 'cardiogenic mixing'. It has been calculated that it increases the effective diffusion coefficient by a factor of five.

It occurred to us that we were in the unique position to be able to study the effect of pulmonary

Table 1 Pulmonary venous P_{O} response to a step increase in $F_{\text{I}\text{O}_2}$ during pulsatile and non-pulsatile perfusion (normal lungs)

| Expt | $\Delta P / \Delta t$ (kPa/s) ^a | | | | Time to take-off ^b (sec) ^b | | | |
|-------|--|----|---------------|---|--|----|---------------|---|
| | Pulsatile | n | Non-pulsatile | n | Pulsatile | n | Non-pulsatile | n |
| 5/11 | 2.6 | 8 | 1.5 | 6 | 10.4 | 8 | 11.6 | 6 |
| 11/11 | 2.77 | 6 | 1.56 | 6 | 10.3 | 6 | 10.5 | 6 |
| 3/17 | 3.40 | 9 | .90 | 6 | 7.0 | 9 | 9.8 | 6 |
| 17/12 | 2.33 | 8 | .90 | 6 | 10.1 | 8 | 6.1 | 6 |
| 25/1 | 3.09 | 9 | 3.56 | 9 | 8.8 | 9 | 10.1 | 9 |
| 27/1 | 5.68 | 13 | 4.68 | 9 | 6.0 | 13 | 5.8 | 9 |
| 1/3 | 3.63 | 8 | .85 | 6 | 6.8 | 8 | 1.0 | 6 |
| 3/5 | 3.67 | 9 | 3.83 | 6 | 6.1 | 8 | 6.2 | 6 |
| 12/5 | 3.06 | 9 | .97 | 9 | 7.0 | 9 | 8.6 | 9 |
| 13/5 | 1.59 | 9 | .59 | 9 | 7.8 | 8 | 9.8 | 9 |
| 13/5 | 1.75 | 3 | .46 | 3 | 8.3 | 3 | 9.5 | 3 |
| 2/6 | 2.77 | 6 | 2.43 | 6 | 7.3 | 6 | 7.8 | 6 |
| 9/6 | 1.49 | | 1.88 | 4 | 8.9 | | 10.0 | 4 |
| 21/9 | 1.01 | 9 | 0.80 | 6 | 12.6 | 9 | 19.0 | 6 |
| 6/10 | .15 | 9 | 1.21 | 6 | 1.4 | 9 | 13.6 | 6 |
| 20/9 | 2.31 | 9 | 1.73 | 6 | 7.0 | 9 | 8.4 | 6 |

$\Delta P / \Delta t$ is the slope of the middle 1/3 of the recorded pulmonary venous P range. Values are the mean from observations.

The time from switching to a high $F_{\text{I}\text{O}_2}$ (0.96) to start of the rise in venous P . Values are the mean of observations.

Table 2 Pulmonary venous P_{O} response to a step increase in $F_{\text{I}\text{O}_2}$ during pulsatile and non-pulsatile perfusion (edematous lungs)

| Expt | $\Delta P / \Delta t$ (kPa/s) ^a | | | | Time to take-off ^b (sec) ^b | | | | ΔW (g) ^c |
|-------|--|---|---------------|---|--|---|---------------|---|-----------------------------|
| | Pulsatile | n | Non-pulsatile | n | Pulsatile | n | Non-pulsatile | n | |
| 2/6 | 2.03 | 4 | 1.93 | 4 | 9.1 | 4 | 9.6 | 4 | 7 |
| 22/9 | 1.34 | 3 | 0.91 | | 10.1 | 3 | 18.0 | 2 | 3 |
| 27/10 | .62 | 4 | 1.86 | 3 | 8.1 | 4 | 8.4 | 3 | 10 |
| 12/11 | 3.69 | 4 | 2.91 | 4 | 7.5 | 4 | 9.5 | 4 | 6 |
| 17/11 | .42 | 4 | 2.34 | 3 | 8.3 | 4 | 8.5 | 4 | 10 |

$\Delta P_{\text{O}} / \Delta t$ is the slope of the middle 1/3 of the recorded pulmonary venous P_{O} range. Values are the mean from observations.

The time from switching to a high $F_{\text{I}\text{O}_2}$ (0.96) to start of the rise in venous P . Values are the mean of observations.

ΔW gives the amount of accumulated extra vascular fluid caused by left atrial pressure elevation.

Table 3 Velocity of O_2 advancement in non-ventilated lungs during pulsatile and non-pulsatile perfusion

| Expt | | Time from air/oxygen gas switch till the rise in | | | | Flow (ml/min) |
|------|---------------|--|---|------------|---|---------------|
| | | P trace (s) | n | P (s) | n | |
| 6/9 | Pulsatile | 4.2 ± 0.45 | 5 | 4.3 ± 1.1 | 8 | 12 |
| | Non-pulsatile | 4.3 ± 0.69 | 6 | 21.9 ± 3 | 6 | |
| 13/9 | Pulsatile | 4 ± 0.49 | 6 | 19.8 ± 2.1 | 6 | 180 |
| | Non-pulsatile | 3.0 ± 0.96 | 6 | 10.8 ± 1.7 | 6 | |
| 20/9 | Pulsatile | 3.8 ± 0.19 | 4 | 29.0 ± 2.8 | 6 | 300 |
| | Non-pulsatile | 4.3 ± 1.33 | 6 | 18.5 ± 1.9 | 8 | |
| 27/9 | Pulsatile | | | 23.2 ± 4.3 | 9 | 300 |
| | Non-pulsatile | | | 18.3 ± 1.7 | 6 | |

Skin blood flow in humans as a function of environmental temperature measured by ultrasound

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We have measured the blood velocities in arteries supplying the skin in humans both in areas with arteriovenous anastomoses (AVA) and in skin areas without AVA in different temperature situations by means of pulsed bidirectional doppler ultrasound instrument. The instrument measures the time average of the instantaneous mean of the blood velocities present in cross-section of vessel. So long as the cross-sectional area of the vessel is constant, this average velocity is proportional to volume flow. We observe rapid and large fluctuations in blood velocities in arteries supplying skin areas with AVA in a comfortably warm environment. These fluctuations are substantially larger and more rapid than described by previous authors who have used plethysmograph methods. The blood velocities are more constant both at higher and low environmental temperatures. There are no similar fluctuations in blood velocity in arteries supplying skin areas without AVA.

Key words: Skin blood flow, temperature, ultrasound, doppler.

Circulation has long been of interest to biologists because of its dominant role in temperature regulation (Fredenq 1882). Since each species has its own unique temperature situation, human skin circulation has to be manipulated in humans (Elizondo 1977). This limits possible experimental methods. Temperature measurements (Stewart 1911, 1912, Lewis 1930) and plethysmograph method (Brodie & Russell 1951, Hewlett & Van Zwilunenburg 1909) have almost widely used.

With venous occlusion plethysmography one can measure blood flow to the extremities or to parts of them, as for instance flow to hands or feet. In the total, the skin circulation accounts for about 60% of the total blood flow, and even more in warmer situations (Greenfield, Shepherd & Whelan 1951). The skin vascular anatomy in the hands and feet is different from that in other parts of the body (Gross 1902). There are a great number of shunts between small arteries and veins in the dermis, the so-called arteriovenous anastomoses (AVA). The

AVA are nervously regulated and make possible great changes in skin blood flow (Clark 1938, Molyneux 1977).

Earlier authors all agree that the nervous control of AVA plays an important role in temperature regulation (Molyneux 1977, Hellon 1971, Hertzman 1959).

The presence of AVA in different skin areas in humans are as follows: there are AVA in the nailbed of fingers and toes, in the fingertip and sole, in the palmar aspects of the fingers, in the thenar and hypothenar eminences of the hand, but not on the dorsum of the hand or fingers (Grant & Bland 1931). In addition AVA have been shown in the external ear (Prichard & Daniel 1956), but not in any other skin area in humans. Molyneux (1977) claims that Hoyer (1877) has found AVA on the tip of the nose in humans, but such a statement has not been made by Hoyer.

The main contribution to the study of skin blood flow in the extremities has been made by Burton & Taylor (Burton 1939, Burton & Taylor 1940). The

vascular pulsations in the absence of the heart. Since the branches of the bronchial and the arterial tree run in parallel and in close proximity to each other it was a distinct possibility that the phenomenon of cardiogenic mixing were at least in part due to arterial pulsations. In order to keep convective gas movement low the lungs were not ventilated in this series of experiments. We found no systematic difference in the velocity of the oxygen front movement from the trachea to the venous blood when the two modes of perfusion were compared. Tracheal air was continuously sampled with a flow of 70 ml/min via a capillary catheter. From the sampling point to the alveoli however a situation of nearly pure diffusion plus or minus arteriogenic mixing was created. If the vascular pulsations enhance the effective diffusion coefficient with a factor of five or even much less a difference should have been detected.

Cardiogenic mixing therefore appears to be the result of a direct action of the heart on the lungs rather than an indirect effect via the vascular bed. This conclusion is in agreement with Engel et al. (1973) whose work indicates "that pulsations of blood vessels next to or beyond the airways did not constitute an important mechanism. Although such an accordance in a difficult and controversial field is nice it leaves us without any obvious explanation of the behaviour of the elusive variable 'time to take-off'".

We conclude on the basis of the slope of the pulmonary venous P_{50} curve that in normal lungs pulsatile and non-pulsatile perfusion is equally efficient when gas exchange is concerned and that pulsatile flow is of advantage in edematous lungs.

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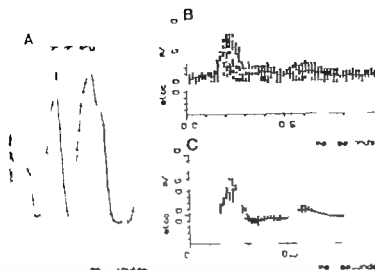


Fig. 2 Fluctuation in blood velocity in the right ulnar artery in a 77-year-old male subject in a comfortably warm environment ($24-25^{\circ}\text{C}$). (A) Time average for each cardiac cycle of the cross-sectional areas of the blood flow in the vessel as a function of time. (B) Blood velocities present in the vessel as a function of time during one cardiac cycle (marked B in panel A). The instantaneous cross-sectional areas are plotted as continuous lines. (C) Blood velocities present in the vessel as a function of time during one cardiac cycle (marked C in panel A).

presented at a meeting of The Scandinavian Physiological Society (Thoresen & Wallae 1978).

METHODS

The Doppler velocitymeter UNIDOP used in the present investigation and the computer systems for data analysis have been described in detail elsewhere (Hatcheland & Wiles 1980; Wiles 1977).

In this investigation UNIDOP is used in the pulsed mode with an operating frequency of 6 MHz. The depth of penetration is from 0.2 to 2.5 cm. All the arterial vessels are situated within this range. The transducer chosen for each artery had a diameter approximately 1.5 times greater than the vessel diameter. With this diameter of the transducer a small cross-section of the vessel is illuminated with a reasonably constant intensity (Guddoy et al. 1980). The angle between the direction of the soundbeam and the vessel axis is kept at about 45° .

During measurements, the velocity spectra were calculated each 16 ms and displayed continuously in real time on a scope. The instantaneous mean velocity or the time average of this mean velocity over each cardiac cycle could be displayed on a storage scope or on an x-y plotter connected to the computer.

The experiments were performed on 6 subjects, 4 males and 2 females (22–40 years) without any known vascular disease. During the experiment, the subject was wearing a suit of modern cut and lying in a quiet room in one of two temperature variations, cold, $15-18^{\circ}\text{C}$, comfortably warm, $24-25^{\circ}\text{C}$ or warm $41-43^{\circ}\text{C}$. The air humidity was

always low. The subject had been resting in the room for at least 30 min prior to the measurements.

The velocity measurements were performed on arteries supplying different skin areas: the superficial temporal artery supplying the forehead, a small artery on the tip of the nose, a small artery in the earlobe, the lateral thoracic artery at the height of the nipple, different small arteries supplying the skin of the lower abdomen, the knee and elbow region, the ulnar and the radial artery at the wrist, the radial 3rd finger artery at the base of the 1st phalanx, the posterior tibial artery behind the medial malleolus and the lateral artery of the 1st toe at the base of the 1st phalanx.

RESULTS

Fig. 2A shows the average blood velocity for each cardiac cycle in an ulnar artery in a comfortably warm situation. There are three time periods with a rapid fall in average mean blood velocities. We interpret such falls in velocities as corresponding to vasoconstrictions. In two of the periods, the blood velocity decreases rapidly within 3–5 heartbeats to a velocity-value only 1/10 of the starting value. The constriction is followed by a somewhat slower dilatation. Panel B in Fig. 2 shows the velocity spectra and the instantaneous mean velocity of one heartbeat on top of the first vasodilatation period.

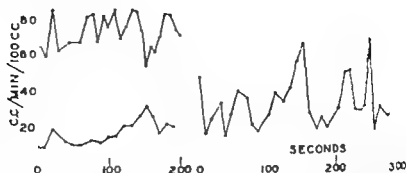


Fig. 1. Burton's measurements of fluctuations in the blood flow in a finger in the high and low flow range (plotted to the left) and in the medium flow range (plotted to the right) measured each 10 s by means of a venous occlusion plethysmograph (from Burton (1939) his Fig. 7 by kind permission of The American Physiological Society)

skin blood flow in other parts of the human body has not been studied in comparable detail due to the absence of suitable methods.

Burton describes fluctuations in blood flow in the finger measured with a finger plethysmograph (Burton 1938). The periodic fluctuations were seen to take place synchronously in all fingers and toes. Burton's main findings are displayed in Fig. 1 which is reprinted from his paper (1939). In Burton's own words: 'Physical regulation of body temperature is accomplished not by the setting of peripheral blood flow to an appropriate steady level but by the adjustment of the average of a flow which is fluctuating rhythmically between high and low values. The rhythm is markedly modified by the conditions of temperature regulation. The amplitude of the waves is greatest in the middle range of blood flow and of temperature is less in dilatation and least in constriction. The frequency of the rhythm is also modified, the faster rhythms being associated with cooler conditions and with lower average values of the flow.' As shown in Fig. 1, 10 s elapse between each of Burton's measurepoints. By this method it is difficult to increase the time resolution above this value. There are also other limitations and difficulties connected with the plethysmograph method. Burton (1939) claimed that the minimal and maximal volume flow values in a finger are of the order of 1 and 90 ml/min/100 ml tissue. The plethysmograph however cannot measure very high or very low flow values (Abrahamson 1967; Greenfield & Shepherd 1950). Burton's own measurements (Fig. 1) indicate a low flow value of 17 ml/min/100 ml tissue compared to a high flow value of 70 ml/min/100 ml tissue.

Because of the technical design of the venous

occlusion plethysmograph only parts of the extremities have been investigated. Consequently there is an almost complete lack of information about the corresponding flow variations in all areas in other parts of the body.

To reinvestigate the skin blood flow pattern in the extremities by a method possessing better time resolution and to extend the skin blood flow measurements onto other parts of the body we have used a bidirectional pulsed doppler ultrasound system developed in our laboratories (Hartelund, Enksson 1980; Wille 1977; Guldvog et al. 1980). The ultrasound field emitted from the transducer is a width sufficient to illuminate the total cross-section of the vessel with approximately constant intensity. The ultrasound equipment measures the instantaneous mean velocity across the lumen of the vessel. As long as the cross-sectional area of the vessel is constant, this mean velocity is proportional to volume flow. The last assumption can of course be questioned and we will return to this point in the discussion.

With this method we have explored the time course of the fluctuating flow pattern in the skin in greater detail. Our findings are mainly in accordance with the results reported by Burton & Taylor (1940). We find however that the fluctuations in skin flow distal in the extremities are substantially greater and more rapid than could possibly have been detected by use of the plethysmograph method. In skin areas without AVA on the trunk and head the findings were different. The blood flow to these areas did not show any signs of the flow fluctuations so characteristic of skin areas with AVA.

A preliminary report

of the results

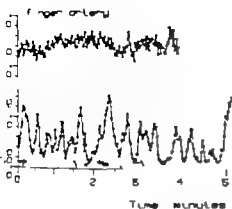


Fig. 5. Blood velocity in an artery to the third finger in 3 positive situations in female subject 26 years old.

plete vasodilation, with high velocity values, only small fluctuations. The velocities are 40% greater than in a cold situation.

Recordings from the posterior tibial, the toe and finger arteries showed the same increase and the same pattern of fluctuations with increasing temperature.

The upper panel in Fig. 4 shows the same three recordings on the ulnar artery in another subject. This subject had a low threshold for sweating, and started profusely during the high temperature recording. The fluctuation pattern persisted in the hands and feet throughout the experiment and the velocity values in this subject were only 8% greater in a warm situation than in a cold situation.

The lower panel of Fig. 4 shows two recordings in a lateral trunk artery in the same subject. The first tracing is from a comfortably warm situation. The velocities show no fluctuations. The upper tracing shows recording from a high temperature situation. There are still no fluctuations and the velocities are only 3 times greater than in the comfortably warm situation. Velocity values obtained in the trunk artery in a cold situation, were only slightly lower than in the comfortably warm situation (approximately 0.015 ml/s).

Other skin areas which do not contain AVA (e.g. forehead, lower abdomen, knee and elbow region), displayed the same nonfluctuating pattern in all temperature situations and only a moderate increase in velocities (4-6 times) as the temperature in the surroundings increased from 18° to 42°C.

Fig. 5 shows recordings from a finger artery of

third subject in the 3 different temperature situations. This person was sweating only slightly in the warm situation.

All our non-sweating subjects showed occasional short periods of vasoconstriction in the warm situation. Typically there was one vasoconstriction every 3 to 7 min with a 40-50% reduction in a cage mean blood velocity in hand and foot arteries.

Measurement performed on a small artery in the tip of the nose showed the same periodic fluctuations in blood velocities as seen in skin areas with AVA.

Fig. 6 shows the results from 3 experiments, which all illustrate the nervous influence on the vascular bed in skin areas with AVA.

In Fig. 6A the recording is from a finger artery during a 9 min period. The subject came in from the cold with continuous vasoconstriction which ceased as he got warmed and the typical fluctuations appeared.

Fig. 6B shows the result of an experiment on an ulnar artery where the subject puts the opposite hand in cold water for 40 s. The average mean velocity decreases immediately to a minimum value and the fluctuating pattern is restored with a delay of 20 s after the hand is taken out of the cold water. This rapid response is known to depend upon intact sensory nerves in the immersed hand and on intact efferent sympathetic nerves to the other (Lewis 1930).

When similar experiments were performed while measurements were being obtained on arteries to the skin of the trunk, no change in blood velocities were observed.

Several other vasomotor reflexes affect skin blood vessels in the hands and feet (Kunkels, Stead & Wers 1939; Abrahamson & Ferns 1940; Burton 1939). The importance of quiet and relaxed environments is shown in Fig. 6C where the subject became aware of a girl watering some plants in the neighbouring hothouse. Within a few seconds his ulnar artery showed a rapid vasoconstriction followed by slower vasodilation.

During recordings we often experienced that disturbances such as phone calls or people moving in the room caused vasoconstrictions and stopped the fluctuating pattern in hand and foot arteries. Trunk arteries did not show this response.

Most of the subjects usually fell asleep during the long experiments in the comfortably warm situation. This did not change the cyclic variation in skin blood velocities.

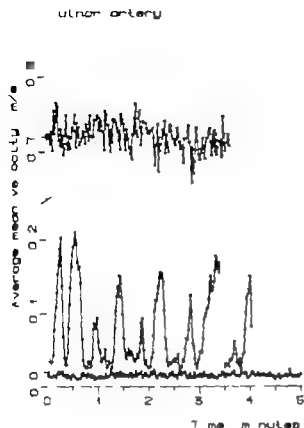


Fig 3 Blood velocity in the right ulnar artery as a function of time in three different temperature situations in the same subject as in Fig 2. Axes and symbols as in Fig 1.

marked B in Fig 2A. There is a forward flow through the total cardiac cycle and there is a continuous great flow in the diastole. Four heartbeats later the average velocity has decreased to 10% and the velocity spectra have changed dramatically as shown in Fig 2C. The maximum velocity during systole is only slightly reduced, the great change being in the diastole. The forward flow in systole is followed by oscillating backward and forward flow during diastole due to the increased peripheral resistance. Usually about 80% of the velocity reduction occurred within 3 heart cycles.

Fig 3 shows three experiments performed on the right ulnar artery on the same subject in 3 different temperature situations.

The lower tracing is from a cold situation, the subject lying lightly clothed in 18°C. The average mean velocity calculated for each successive cardiac cycle is very low and shows only minor variations. The velocity spectra showed diastolic backflow. Such results were always obtained in a cold situation.

The middle tracing is typical for medium warm

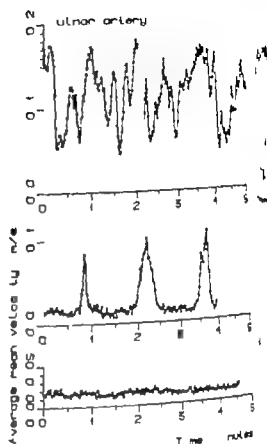
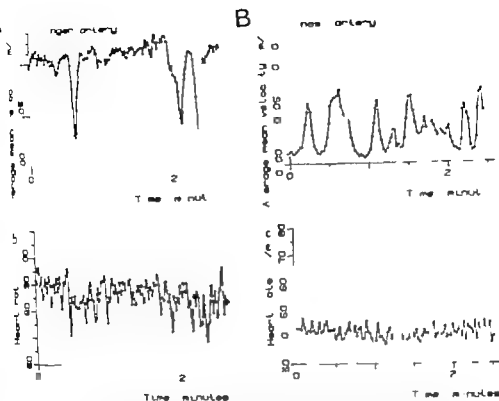


Fig 4 Upper part: Blood velocity in the right ulnar artery in three different temperature situations in a male subject 40 years old. Lower part: Blood velocity in an artery in the skin of the thorax in the same subject in the same three temperature situations shown above (medium and high temperatures).

environments with 2-3 periods of vasodilatation per minute, each having a velocity amplitude 5-10 times greater than the minimal blood velocities. As temperature increases, the frequency of vasodilatations usually decreases, each period lasting longer. Also the velocity values found during vasodilatations increase in value. The result is a decrease in the amplitude of the velocity fluctuations.

The upper tracing is from an uncomfortable warm situation. The subject has rested in a room at 42°C for 2 hours, but he is not sweating. There



7 A, upper panel: Blood velocity in finger artery in female subject, 21 years old, in fairly warm environment. Lower panel: Simultaneous recording of the heart rate calculated by the computer from the ECG recording. Each cycle plotted. B, upper panel: Blood velocity in an artery on the tip of the nose in male subject, 29 years old, in comfortably warm environment. B, lower panel: Simultaneous recording of the heart rate.

section of the vessel each cardiac cycle. If the cross-sectional area of the vessels does not change during the cycle, changes in average velocity values are proportional to changes in volume flow. In previous work (Guldvog et al. 1980; Hauge et al. 1980) we have shown that this condition probably is fulfilled within measurement accuracy in the femoral and common carotid artery in the dog and in the radial carotid and the vertebral artery in man.

What the relationship between the mean velocity and volume flow in the smaller arteries investigated in the present study will be is not apparent. There are two possible mechanisms which could change the diameter of the vessels: (1) The transmural pressure of the vessel could alter due to changes in the vascular resistance peripherally to the measurement point. (2) The nervous output which affects the resistance vessels could also affect the muscular tension where we are measuring. These two mechanisms would probably operate in opposite

directions. If the first mechanism is important, transmural pressure should drop during periods with vasodilatation. The results would be a decrease in vessel diameter and an increase in mean velocities across the vessel lumen greater than corresponding to the increase in flow. If the second mechanism is important, the vessel diameter would decrease during periods with vasoconstriction. The result would be fluctuations in average mean velocities smaller than the corresponding fluctuations in flow. We tend to believe that if either of these mechanisms are of importance, it is more likely to be the second one than the first. The reason for this belief is that the vascular resistance between the aorta and some of the vessels where we are measuring (the ulnar, the radial and the posterior tibial arteries) must be small. It is difficult therefore to imagine large changes in transmural pressure in these vessels, when the central blood pressure is constant. Our conclusion is therefore that neither the observed fluctuations in average mean blood ve-

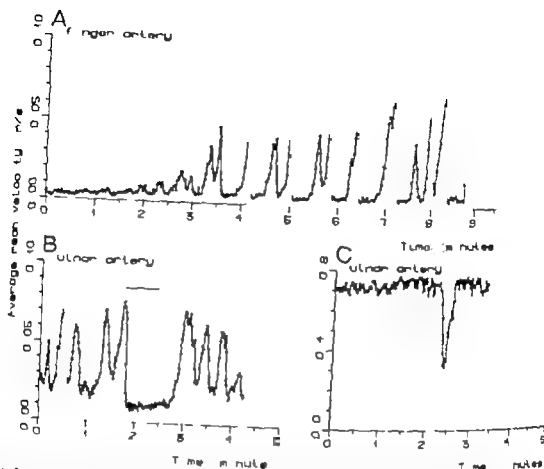


Fig. 6. (A) Blood velocity in a finger artery in a period with increasing temperature. Male subject 79 years old. (B) Blood velocity in the right ulnar artery in a male subject 26 years old in a medium temperature situation. The subject put the opposite hand in cold water for 40 s. (C) Blood velocity in the right ulnar artery in a male subject 27 years old in a warm environment. The vasoconstriction is caused by the sight of a girl in the neighbouring hothouse.

Measurements performed simultaneously on the two radial arteries of one subject showed synchronous fluctuations as described by Burton (1939).

In one experiment on one of us (L. W.) we measured the radial intraarterial blood pressure simultaneously with measuring blood velocity on the opposite 3rd finger in a comfortably warm situation. In contrast to Burton we did not find any systematic changes in the blood pressure corresponding to changes in velocities.

DISCUSSION

The quantitative knowledge of skin blood flow is mainly based on plethysmograph measurements of limbs and appendages which can be enclosed in a plethysmograph. In the digits the flow goes primarily to the skin, and Burton & Taylor's work (Burton 1939; Burton & Taylor 1940) on finger blood flow has supplied most of the information about the amount and variability of skin circulation.

In the main, our findings confirm Burton's work. However, we find more rapid and greater variations in the blood flow to the hands and feet, probably due to the higher time resolution and flow rate resolution obtained by our method.

In certain skin areas mentioned in the introduction there are arteriovenous anastomoses which permit a great blood flow to pass directly from arteries to veins if they are open. We find that the skin areas displaying a fluctuating pattern in flow velocities coincide with the anatomical distribution of AVA. The correlation is better than that presented in the figures; for example there are no fluctuations in small arteries on the dorsum of the hand.

As far as we know, no anatomical investigations have documented AVA in the tip of the nose (Cliff 1938). However, our results suggest that there may also be AVA in this skin area, since we observe a fluctuating velocity pattern which changes with environmental temperature (Fig. 7B).

We measure the average velocity through

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locities nor the changes seen in velocities from one temperature situation to another overestimate the corresponding fluctuations and changes in flow.

Measurements of the ulnar, finger, posterior tibial, toe and nose arteries show rapid fluctuations in skin blood flow in a comfortably warm situation. The maximal frequency of fluctuations which we have observed is 6 per minute. The frequency decreases as the temperature increases. With increasing temperature the pattern shifts from that of periodic vasodilations on a steady vasoconstriction to periods of vasoconstrictions on a high flow vasodilator situation.

The fluctuations are rapid. The average mean velocity may decrease to 10% within 3-4 heart cycles (Fig. 7) and the amplitudes are 3 times greater than Burton (1939) describes them.

The amplitudes get smaller as the temperature increases. This phenomenon was also described by Burton (1939).

The 6 subjects we have investigated showed two different patterns of responses to high temperatures. The non-sweating or slightly sweating persons increased their average mean velocity in hand and foot arteries to 70-40 times the values obtained in a cold situation. They also showed an almost complete cessation of the fluctuating pattern.

On the other hand, in persons who sweated profusely the fluctuating pattern sustained and there was only an 6-8 times increase in the average velocity values. Thus, the pattern of velocities found in the warm situations (42°C) showed considerable differences between persons. Each person was however consistent in his or her reaction to different temperatures. This interindividual variation probably corresponds to the substantial individual variation in sweating patterns described by Heitzman (1957).

Anatomical investigations show AVA to be present in the human ears (Prichard & Daniel 1956). Because of practical difficulties in obtaining measurements on the small arteries in the ear lobe it has not been possible to record for sufficient periods of time in all temperature situations. On the basis of a few recordings our impression is that the ear is the first part of the skin surface to respond with vasodilatation when the person is in the need of heat loss. Thus, there seems to be only a small temperature zone where there is a fluctuating flow pattern in the ear.

The skin of the head, trunk and proximal parts of

the limbs do not contain AVA. The flow response in these skin areas to different environmental temperatures is quite different from the responses in the hands and feet. The pattern is non-fluctuating in all temperature situations. In a comfortable warm situation the velocity values measured on a foot artery are usually not more than twice the value obtained in a cold situation. In the heat stress situation the velocity values increased not more than 3-5 times as compared to the comfortable warm situation. The non-sweating persons again show the greatest increase. This moderate increase is in contrast to the great blood flow values during sweating given by others (Roddie, Shepherd & Whalen 1955; Greenfield 1963).

The plethysmograph has great limitations in flow situations (Greenfield & Shepherd 1964). Burton claims that vasoconstriction is accompanied by a simultaneous increase in the cardiac rate (Burton 1939, his Fig. 8 C and 17). When the plethysmograph fails, he then uses the periods of cardiac acceleration as a measure of the number of vasoconstrictions. We have not in any of our experiments been able to confirm Burton's claim. Indeed, we have not found any systematic relationship between vasoconstriction and heart rate at all. Two examples are shown in Fig. 7. Fig. 7A displays velocities in a finger artery in a fairly warm environment and the corresponding heart rate. The fluctuations in the heart rate, but they are not correlated to the three vasoconstrictions in the finger artery.

Fig. 7B shows the velocities in an artery on the tip of the nose in a comfortably warm environment and the corresponding heart rate. In this person the heart rate is much less variable. There are no correlation between changes in velocities and heart rate.

Neither have we found systematic changes in skin flow connected with respiration as claimed by Burton and others (Burton 1939; Burch, Cohen & Neumann 1941). Finally we found no such systematic changes in blood pressure corresponding to vasodilation and vasoconstriction as claimed by Burton (1939).

It appears from our findings that the fluctuations in blood flow to skin areas with AVA are not correlated to any other cardiovascular parameters or events observed. It appears probable that the marked fluctuations are related to synchronous openings and closures of AVA.

low threshold facilitation of inspiration by lung volume increments

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phly mediated, volume dependent excitatory effect of the phrenic nerve activity has been recently described in anesthetized dogs and pigs, but could be ascertained in rabbits and baboons (Bartoli et al 1975; Hucznak et al 1977; Cross et al 1980; Marzinski et al 1980). Results presented here on a baboon and further characterize a volume dependent facilitatory effect on inspiratory intercostal phrenic activity in this species.

In pentobarbitone anesthetized cat, the movement of mass activity of phrenic (Pfr) and external intercostal (EI) nerves was recorded simultaneously with tidal volume (V), tracheal pressure airflow (V) and end-tidal P_{CO}₂. The effects of changes in lung volume, P and V on the Pfr and EI activities were studied both during spontaneous breathing and after paralysis with gallamine while slowly inflated. Lung movements were varied incrementally for a single breath by means of a modified "servo-respirator" operated at different gains (volume/phrenic ratio) or by phrenic nerve stimulations of different time courses and intensities and, during spontaneous breathing, by chest occlusion at end-expiration. Control and test trajectories were compared at the same level of neural drive.

In lightly anesthetized cats, increments in lung volume produced a facilitatory effect on both Pfr and EI. Since this effect was completely abolished by bilateral cervical vagotomy, it apparently did not involve chest wall receptors. The facilitatory response, expressed in terms of the arithmetic difference in amplitude of test and control trajectories (Δ Pfr and Δ EI, respectively) was linearly related

to the corresponding difference in lung volume (ΔV) throughout the inspiratory phase, but was not similarly related to changes in flow (ΔV). This is evident from Fig. 1 where Δ Pfr and Δ EI are seen to increase progressively with increasing ΔV during inspiration while the difference in flow remained constant. Fig. 1 further illustrates that the responses appear at volume and pressure changes well below eupnoeic V and P.

Fig. 1 shows that this volume dependent facilitatory effect could also be demonstrated at zero values of V occurring in the inspiratory phase. Two

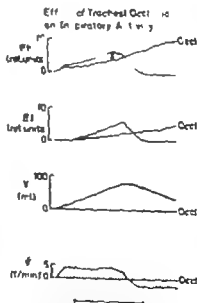


Fig. 1. Respiratory facilitation during spontaneous breathing. From above: "Integrated" phrenic (Pfr), external intercostal (EI) activity, volume (V) and flow (V). The computer average of 5 test trajectories (tracheal occlusion at end-expiration) is superimposed on the preceding control trajectories. Five breaths elapsed between each control/test pair. 2 represent Δ Pfr (see text for further explanation).

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Low threshold facilitation of inspiration by lung volume increments

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Lowly mediated, volume dependent excitatory effects on phrenic nerve activity have been recently described in anesthetized dogs and pigs, but could not be ascertained in rabbits and baboons (Bartoli et al. 1973; Heszczuk et al. 1977; Cross et al. 1980; Mrozowski et al. 1980). Results presented here confirm and further characterize a volume dependent facilitatory effect on inspiratory intercostal diaphragm activity in this species.

In 11 pentobarbitone anesthetized cats, the moving average of mass activity of phrenic (Phr) and lateral intercostal (EI) nerves was recorded simultaneously with tidal volume (V), tracheal pressure, airflow (V), and end tidal PCO₂. The effects of changes in lung volume (P and V) on the Phr and EI activities were studied both during spontaneous breathing and, after paralysis with gallamine, while artificially ventilated. Lung movements were varied electronically for single breaths by means of a micro driven servo-respirator operated at different gains (volume/phrenic ratio) or by phrenic triggered inflations of different time courses and amplitudes and, during spontaneous breathing, by aortic occlusion at end-expiration. Control and test trajectories were compared at the same level of neural drive.

In lightly anesthetized cats, increments in lung volume produced a facilitatory effect on both Phr and EI. Since this effect was completely abolished by bilateral cervical vagotomy, it apparently did not involve chest wall receptors. The facilitatory response, expressed in terms of the arithmetic difference in amplitude of test and control trajectories (Δ Phr and Δ EI, respectively) was linearly related

to the corresponding difference in lung volume (Δ V) throughout the respiratory phase, but was not similarly related to changes in flow (Δ V). This is evident from Fig. 1 where Δ Phr and Δ EI are seen to increase progressively with increasing Δ V during inspiration while the difference in flow remained constant. Fig. 1 further illustrates that the responses appear at volume and pressure changes well before expiratory V and P.

Fig. 1 shows that this volume dependent facilitatory effect could also be demonstrated at zero values of V occurring in the inspiratory phase. Two

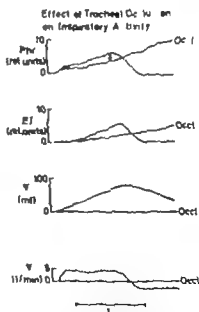


Fig. 1 Respiratory facilitation during spontaneous breathing. From above: integrated phrenic (Phr), external intercostal (EI) activity, volume (V) and flow (V). The computer average of 5 test trajectories (tracheal occlusion at end-expiration) is superimposed on the preceding control trajectories. Five breaths elapsed between each control/test pair. Δ represents Δ Phr (see text for further explanation).

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Effect of end expiratory pressure

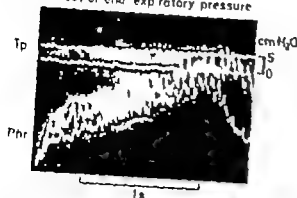


Fig. 2. Effect of positive shift of tracheal pressure. Two superimposed phrenic trajectories, each while the respirator was switched off. Lower trajectory at zero tracheal pressure and upper at +5 cm H₂O. Several normal breaths elapsing between trajectories.

Effect of Pressure Changes Produced by Different Gains on Ph and EI

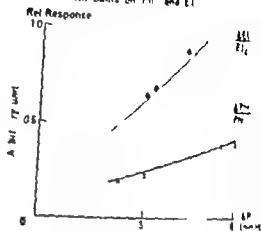


Fig. 3. Maximal response values relative to phrenic and external intercostal control activity (Phr EI) plotted against changes in tracheal pressure.

phrenic trajectories during breaths in which the "servo-respirator" is turned off (gain 0) are compared at zero pressure (lower curve) and with constant positive pressure applied in the preceding expiratory phase and maintained throughout the inspiratory phase (upper curve). Pressure increments during the expiratory phase had no excitatory effects. ΔPhr remained almost constant independent of inspiratory time and increasing Phr .

A linear stimulus/response relationship was also obtained by changing the gain of the servo-respirator to produce different volumes and relating the maximal response value of ΔPhr and ΔEI between breaths to the simultaneous ΔV . For purposes of comparison between the phrenic and intercostal responses, ΔPhr and ΔEI were normalized to Phr and EI trajectories at a gain setting which produced a V_T similar to spontaneous breathing (as seen in Fig. 3). Any given ΔP produced a larger percentage change in $\Delta EI/EI$ control compared to $\Delta Phr/Phr$ control. Similar results were obtained using phrenic triggered inflation of different shapes and delays in onset. With this procedure it was further demonstrated that V per se did not act as an excitatory stimulus for this response.

The volume dependent inspiratory facilitation was consistently decreased or abolished by small additional doses of pentobarbitone (2–4 mg/kg). After vagotomy the effect could be reproduced in Phr and EI by electrical vagal stimulation at similar threshold strengths required to produce shortening of inspiratory duration (i.e. the classical Hering-Breuer reflex).

From these results we conclude that the threshold lung mechanoreceptors which monitor volume related information can facilitate inspiratory activity during eupnoea, increasing the stronger facilitatory effect exerted on intercostal motoneurons compared to phrenic motoneurons to progressively increase the relative contribution of the intercostal muscles to the production of tidal volume—an effect which would increase in importance as higher tidal volumes are demanded.

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In vitro studies on a two-pool storage of adrenaline and noradrenaline in granule material from bovine adrenal medulla

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UVNÄS B. & ÅBORG C. H. In vitro studies on a two-pool storage of adrenaline and noradrenaline in granule material from bovine adrenal medulla. *Acta Physiol Scand* 1980; 109: 345-354. Received 1 Nov 1979. ISSN 0001-6772. Department of Pharmacology, Karolinska Institute, Stockholm, S. edes.

Adrenal medullary granules were isolated by millipore filtration according to Pomeroy & Trifaró (1967) and then lysed in deoxygenated water. In a perfusion system the influence of concentration and pH was studied on the uptake of biogenic amines (PbEA, TA, DA, NA, A, Ach and H) and sodium ions by lysed and by dialyzed material. The results suggest a two-pool storage of A and NA in the granules. A minor pool with cation exchanger properties binds unselectively organic (biogenic amines) and inorganic cations with U_{max} of 400-500 nmol/mg granules dry-weight. This minor pool (pool I) was fully charged at amine concentrations >10 mM. A larger pool binds selectively A and NA—possibly stereospecifically L-NA and L-A— U_{max} 1-100 nmol/mg granules. This larger pool (pool II) required A and NA concentrations ~ 200 -300 mM for maximal filling. I pool CA ions are assumed to be electrostatically linked to carboxyl groups, which become available as hypothetical $COO^- \rightleftharpoons H_2N$ salt bridges are successively forced open by increasing CA concentrations (>10 -30 mM). ATP ions become attached to the concomitantly unmasked NH groups.

Key words: Adrenal medullary granules, storage of catecholamines, noradrenaline, adrenaline, adrenal medulla.

Bovine adrenal medullary granules have a high content of adenosine 5'-triphosphate (ATP) and it was first suggested that the positively charged adrenal catecholamines (CA) were stored ionically bound to the negatively charged polyvalent nucleotide (Blanchard et al 1946, Falck et al 1936). In vitro CA and ATP have a tendency to aggregate into high molecular complexes—especially in the presence of divalent earth metal ions (Ca^{2+} and Mg^{2+}). Bernheim et al (1971) proposed that the formation of mixed high molecular weight aggregates with ATP plays an essential role in the storage of CA in adrenal medullary granules. Various other more or less defined nondiffusible storage complexes containing ATP, Ca^{2+} and Mg^{2+} , proteins and other macromolecules have been suggested (for ref. see Green 1976, Smith & Winkler 1972, Sjöström 1970 and others).

The histamine storing mast cell granules contain no appreciable amounts of ATP but have a high

content of heparin. Since histamine and heparin form a rather stable salt in vitro, histamine was assumed to be stored in the mast cell granules as a histamine-heparin salt. However, in a series of papers (for ref. see Uvnäs 1977) we have presented experimental evidence to show that the mast cell histamine is stored not as a heparin-histamine salt but as ionic linkage to protein carboxyl groups in a heparin-protein complex that forms the matrix of the granules. This granule matrix has the properties of a weak cation exchanger and the release of histamine during the exocytotic process in the mast cell is the result of a cation exchange between the histamine linked to the granule matrix, and extracellular cations, especially sodium ions.

A storage and release process due to cation exchange should be a simple and thereby attractive way of explaining the storage and release also of other electrically charged granule stored components. In a previous paper (Uvnäs & Åborg 1977)

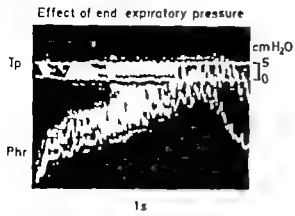


Fig. 2—Effect of positive shift of tracheal pressure. Two superimposed phrenic trajectories, each while the respirator was switched off. Lower trajectory at zero tracheal pressure and upper at +5 cm H₂O. Several normal breaths elapsed between trajectories.

phrenic trajectories during breaths in which the "servo-respirator" is turned off (gain 0) are compared at zero pressure (lower curve) and with constant positive pressure applied in the preceding expiratory phase and maintained throughout the inspiratory phase (upper curve). Pressure increments during the expiratory phase had no excitatory effects. ΔPhr remained almost constant independent of inspiratory time and increasing Phr.

A linear stimulus/response relationship was also obtained by changing the gain of the "servo-respirator" to produce different volumes and relating the maximal response value of ΔPhr and ΔEI between breaths to the simultaneous ΔI . For purposes of comparison between the phrenic and intercostal responses, ΔPhr and ΔEI were normalized to Phr and EI trajectories at a gain setting which produced a V_T similar to eupnoeic breathing. As seen in Fig. 3, any given ΔP produced a larger percentage change in $\Delta\text{EI}/\text{EI}$ control compared to $\Delta\text{Phr}/\text{Phr}$ control. Similar results were obtained using phrenic triggered inflations of different shapes and delays in onset. With this procedure it was further demonstrated that $V_{\text{per se}}$ did not act as an excitatory stimulus for this response.

This volume dependent inspiratory facilitation was consistently decreased or abolished by small additional doses of pentobarbitone (2–4 mg/kg). After vagotomy, the effect could be reproduced in Phr and EI by electrical vagal stimulation at similar threshold strengths required to produce shortening of inspiratory duration (i.e. the classical Hering-Breuer reflex).

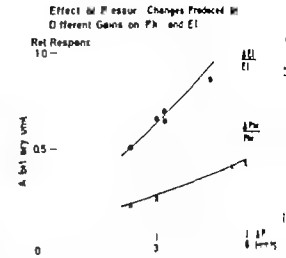


Fig. 3—Maximal response values relative to phrenic and external intercostal control activity (Phr, EI) plotted against changes in tracheal pressure.

From these results we conclude that low threshold lung mechano-receptors, which mediate volume related information can facilitate respiratory activity during eupnoeic breathing. The stronger facilitatory effect exerted on intercostal motoneurons compared to phrenic may function to progressively increase the relative contribution of the intercostal muscles to the production of tidal volume, an effect which would increase in importance as higher tidal volumes are demanded.

This investigation was supported by grant from the Swedish Medical Research Council (Project No. 141444) from Magnus Bergvalls Stiftelse and from Justus Alice Wallenbergs Stiftelse. J. R. R. is indebted to the Royal Swedish Academy of Science for a fellowship and in the agreement for scientific exchange between the Polish Academy of Sciences and the Royal Swedish Academy of Sciences.

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Fig. 1. Electron microscopical picture of granule sediment (9000 g for 10 min). Note the rather homogeneous collection of electron dense membrane-surrounded granules. (365)

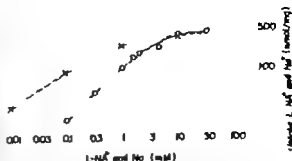


Fig. 2. Concentration dependent uptake of L-NA and N by dialysed granule material with concentrations <30 mM in perfusion fluid, pH 7.4. 4°C. Values taken from Uvna & Åberg (1977) to illustrate uptake by cation exchanger pool (pool 1 in the present paper). — L-NA uptake O—O N uptake

the solution. The samples were then dried (vacuum, 0.01 P₂O₅). The rest of the L-NA-ATP salt solution staining granule material as per on membrane filter (10, 25 mm Dia).

Initially 35 and then 900 KPa psi N₂ gas was applied on a pneumatic compressor. Each row contained 300 µmol of NA and 90 µmol of ATP per oil.

When the solution had perused the filter and only insoluble material remained, this was scraped off (stainless steel spatula) into pre-weighed tube with stopper. The wet weight of material was determined and then the dry weight (after vacuum, NaOH, P₂O₅, 48 h).

To the dried samples and their dried references were added 1 ml of 1 M HCl (and a few glass beads). After sonication by shaking and precipitation of undissolved material at 3000 × g for 30 min, 0.5 ml samples of the supernatant were taken for scintillation counting (Packard, Tri-carb model 3002).

The counting procedures described above allowed calculations of the net uptake of L-NA and of ATP per mg of the dried granule material with corrections for the counts in the moisture of the granule mass.

Effect of pH on the uptake of sodium salt ATP as follows:

900 µmol of NaOH were titrated with ATP (acid) 0.2 mM to pH 7.26 as described above. 0.25 ml of ³H-ATP (50 µCi/ml) and 0.25 ml of ³H-NA (200 µCi/ml) were added and 10 µmol H₂O added to 0.5 ml. 1 ml of sodium-ATP salt solution and 1 ml granule suspension were added and the incubation continued as described above for L-NA-ATP salt. Final concentrations Na⁺ 200 mM, ATP 90 mM.

Concentration dependent uptake of NA

(1) L-NA hydrochloride L-NA hydrochloride was freshly prepared by neutralizing L-NA base (2.538 g)—with an admixture of 0.1 ml of 5.65 N H₂SO₄ and 15 ml of H₂O—concentrated (12 N) HCl. The volume was adjusted to give 600 mM L-NA hydrochloride. Final pH was 6.18. The L-NA hydrochloride solution was diluted with H₂O to give 600, 400, 300, 200, 100, 60, 20, 6 and 2 mM L-NA hydrochloride solutions. 5 ml samples of

these dilutions were added 25 µl of ³H-L-NA (50 µCi/ml) and 25 µl of ³H-Cl (NaCl) (50 µCi/ml). Out of each sample 1 ml was taken and mixed with 1 ml of dialysed granule material. The final concentrations of L-NA in the samples then were 300, 200, 150, 100, 50, 30, 10, 5 and 1 mM. The further procedures are as described above.

(2) L-NA phosphate L-NA phosphate was freshly prepared by neutralizing L-NA base (2.538 g)—with an admixture of 0.1 ml of 5.65 N H₂SO₄ and 15 ml of H₂O—concentrated (12 N) HCl. The volume was adjusted to give 600 mM L-NA phosphate. Final pH 6.14. Dilution with H₂O sampling and admixture of H-L-NA and ³²P⁻ as above with L-NA hydrochloride.

(3) A-ATP salt ATP (acid) was freshly prepared as described above and titrated to pH ~6 with L-NA base. The L-NA-ATP salt solution was diluted with deionized H₂O to give 600 mM solution. The dilutions and the further procedures were as described above for L-NA hydrochloride.

Uptake of PHEA, TA, DA, L-NA and DLA, Ach, H and Na⁺ as determined as described for the uptake of L-NA above. If not stated otherwise all uptake values are given as nmol per mg dried material with deduction of the CA and ATP content.

To minimize possible disturbing effects of various enzymes, of bacterial growth of apomorphine decomposition etc. all procedures described above except titrations were performed in the cold room (4°C). An Agla micro-meter syringe was used for all additions.

The Rastenburg-Korol' id equation is an empirical modification of the Mass Action Law valid for weak cation exchangers (Sørensen 1952). The equation implies that when two cations compete for the same sites of the exchanger plotting the log of the ratios between the concentrations of the two ions in the solution vs the log of the corresponding concentrations in the suspension medium should give a straight line.

$$\frac{[B_1]}{[A_1]} \left(\frac{[A_2]}{[B_2]} \right)^{\beta} = K$$

A₁ and B₁ ions in ion exchange resin. A₂ and B₂ ions in solution. β and K = empirical factors.

we described the ability of a dialyzed and thereby ATP free granule material from bovine adrenal medulla to bind inorganic and organic (biogenic amines) cations. The binding could be ascribed to the cation exchanger properties of the material. The similarity of the uptake curves, the identical uptake maxima and the narrow pH range (between 4–7) for the uptake of all the inorganic and organic cations studied indicated the binding of these ions to common sites, probably carboxyl groups.

The fact that all the uptake curves fitted the Rothmund Kornfeld equation for weak cation exchangers corroborated the cation exchanger properties of the dialyzed material. However, the CA binding capacity corresponded to only 20–30% of the normal *in vivo* content of CA in the bovine adrenal medulla.

In the present study we will describe the ability of medullary granule material to bind additional amounts of CA, the total binding capacity *in vitro* being sufficient to comply with the storage requirements for CA *in vivo*. Our observations indicate the existence—at least *in vitro*—of two storage pools. The smaller of the two pools has the properties of a cation exchanger and binds unselectively inorganic and organic (e.g. biogenic amines) cations. The other pool, the large one, retains selectively—probably stereospecifically—L-NA and L-A, possibly by a combination of ionic and hydrogen bonds. The two pools will in text and figures be denoted pool I and pool II respectively.

METHODS

Preparation of granule material

Bovine adrenal medullary granules were isolated according to Polaner & Trifaró (1967). Adrenals were transported from the slaughter house on ice and the isolation procedure started about 3 h post mortem by homogenization of the medullas in ice-cold 0.3 M sucrose (glass homogenizator). After centrifugation at 800 g for 10 min the supernatant was sucked off and allowed to pass under slight N_2 -pressure (700 kPa) through a series of 9 mm millipore filters mounted in a Sartorius filter apparatus type SM 162.3. The pore sizes consecutively used were 3, 1.2, 0.6, 0.45 and 0.1 μ m. The final filtrate was centrifuged at 70 000 \times g for 10 min. The opalescent supernatant was decanted together with the upper loose superficial sediment layer. The walls of the tube and the pellet surface were carefully dried with filter paper. In the electron microscope the remaining sediment was shown to consist of rather homogenous population of electron dense granules surrounded by a membrane (Fig. 1).

The sediment was lysed in 10 ml of deionized water. After careful stirring of the suspension 0.5 ml was re-

moved for determining the dry weight and 100 μ l for the CA content. The rest of the suspension was used for studies on the uptake of inorganic and organic cations.

For some experiments the lysed material was dialysed against deionized water under continuous rocking for 48 h (4 ml of suspension against 4000 ml of H₂O, ATP procedures described above were performed in the cold room (~+4°C).

Studies on the uptake, storage and release of biogenic and other biogenic amines in mast cell granules can simply be done on sedimental material from lysed granules, since the amine binding granule material remains undissolved. This is not the case with the material from large granules which to a great extent goes into solution by lysis of the granules. Only a minor binding capacity remains in the unsoluble precipitable rest (membranes, etc.) but is far too low to account for the original binding capacity of the granule material. Since the CA binding material of the medullary granules was assumed to be dissolved after lysis of the granules a technique was developed by which uptake studies could be performed on suspended lysed granule material which then was trapped under slight N_2 pressure (500 kPa) on filters selected to allow minor molecules like CA, organic cations, ATP etc. (molecules below ~10 000) in pass but to exclude proteins and other polymers. When still moist the granule material was scraped off and brought into pre-weighed plastic tubes with stoppers. After determining the wet weight the samples were dried for 48 h over NaOH, P_2O_5 in vacuum and the dry weight was determined. The dried material was then analysed for its uptake of biogenic amines, ATP, inorganic cations etc. as exemplified and described in detail below.

Influence of pH on the uptake of L-A

ATP (acid) was freshly prepared by passing a sodium ATP salt (Na ATP 3.5 H₂O MW 614) through a 5% column of Dowex 50W-X2 (11 \times 900 μ m) of Na-ATP were put on the column and eluted with 0.5 ml of deionized water. The content of ATP in the effluent was determined fluorimetrically (since a few per cent were lost during the ion exchange procedure) and the ATP concentration was then adjusted to 700 mM.

10 000 μ m of L-NA (base) was suspended in 5 ml of deionized H₂O containing 0.1 ml of H₂SO₄ (conc.). The freshly prepared ATP (acid) conc. 700 mM was added under stirring until the L-NA (base) had gone into solution at pH ~6. 12.5 ml of the ATP solution corresponding to 100 μ m were consumed to dissolve and acetate.

10 000 μ m of L-NA (base)

0.5 ml of 700 mM ATP (50 μ l/ml) + 25 ml of 91.3% H₂SO₄ (10 μ l/ml) and deionized H₂O to 25 ml were added. Final pH 6.4.

L-NA-ATP salt solution was acidified to desired pH with conc. HCl. One ml sample were taken for uptake studies and 0.5 ml samples for the determination of the dry weight of the solutions (vacuum NaOH, P_2O_5).

One ml of the lysed granule material was mixed with 0.5 ml of the L-NA-ATP salt solution. 0.5 μ l of the mixture was pipetted into pre-weighed plastic tubes (11 \times 900 μ m) with a stopper. The tubes were re-weighed to get the wet weight.

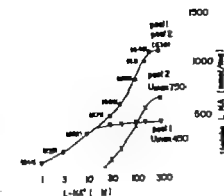


Fig. 4 Graphical construction of two-pool system for the storage of L-NA in dialyzed adrenal medullary granule material (from Fig. 3a). \bullet — \bullet total uptake of L-NA (phosphate) U_{max} 1200 nmol/mg pH indicated by figures above brackets. ∇ — ∇ the initial uptake phase of the elevated pHs calculated according to the Rothmund-Korndorf equation U_{max} 450 nmol/mg pool 1. --- constructed second uptake phase by deduction of initial uptake curve from total uptake curve. U_{max} 750 nmol/mg—pool 2.

tion for a reconstruction of the L-NA uptake at pH 7.4 a typical uptake curve was obtained with a U_{max} of 450 nmol/mg (see Fig. 3a and compare with Fig. 7).

At L-NA concentrations above 10–30 mM the retention of L-NA by the granule material increased further reaching an apparent maximum around 1100–1200 nmol/mg at an L-NA concentration in the perfusion fluid \sim 300 mM. This second phase of L-NA uptake showed other characteristics than the initial one. It was quite resistant to acidification, not being seriously reduced until below pH 6 (for further discussion see page 350). The total uptake of L-NA varied between batches from 800 to 1200 nmol/mg dry material, but was never observed to reach the values required for an uptake corresponding to the normal CA content of the adrenal medulla \sim 2000 nmol/mg wet dry weight (CA and ATP deducted) or \sim 1300 nmol/mg total granule dry weight, as reported by Hallarp (1946).

A graphical separation of the two phases of L-NA uptake is illustrated in Fig. 4. Using the Rothmund-Korndorf equation the initial uptake phase could be completed for L-NA concentrations up to \sim 300 mM and for the pH denoted on the uptake curve. The U_{max} obtained by this calculation was 450 nmol/mg. The deduction of the values thus obtained for the initial uptake phase from those for the

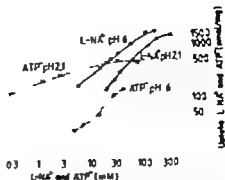


Fig. 5 Concentration dependent uptake of ATP and L-NA by lysed granule material at pH 1 and 6. \bullet — \bullet L-NA pH 6 --- ATP pH 6 --- L-NA pH 1 --- ATP pH 1.

total uptake yielded a curve with a U_{max} of 750 nmol/mg. The two uptake curves suggested to us the existence of two storage compartments in Fig. 4 denoted pool 1 and pool 2 respectively.

Comment. Although the mechanism of storage of L-NA in pool 2 remained to be studied the results obtained with the dialyzed material revealed the interesting fact that the retention of L-NA did not require ATP as anion or as a complexing agent. Fig. 3a and b show an uptake of L-NA both as phosphate and as chloride to about the same U_{max} \sim 1200 nmol/mg. To judge from the retention of administered $^{32}\text{PO}_4$ (Fig. 3a) and ^{36}Cl (Fig. 3b) there was no corresponding uptake of these anions at the pHs used, 6.4–6.7 and 5.1–6.0, respectively.

Lysed—but not dialyzed—granule material

As evident from the above observations dialyzed granule material was able to retain considerable amounts of L-NA (800–1200 nmol/mg) although not as much as stored in the adrenal medulla in vivo (\sim 2000 nmol/mg CA Hallarp 1959). Since dialysis might have reduced the CA binding capacity of the granule material by removing binding components by causing conformational changes of the granule matrix etc. the subsequent studies were performed on lysed—but not dialyzed—material. Since such material contained \sim 6 $\mu\text{mol/ml}$ of endogenous CA, and thereby pool 2 was expected to be more or less filled (see Figs. 2 and 3a) most experiments no attempt were made to record separately the two uptake phases, only the total uptake being determined.

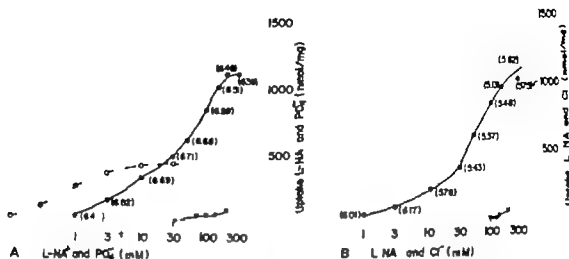


Fig 3a Concentration dependent uptake of L-NA and PO_4 by dialyzed granule material. Total uptake of L-NA (●—●) measured pHs within brackets. Initial phase of L-NA uptake (pool I) reconstructed according to the Rothmund Kornfeld equation for pH 7.4 (○—○). Total uptake of PO_4 (■—■). For details see methods.

Fig 3b Concentration dependent uptake of L-NA and Cl^- by dialyzed granule material at 4°C. Measured pHs within brackets. Uptake of L-NA (●—●). Uptake of Cl^- (■—■). For details see methods.

MATERIALS

L-(7 H) noradrenaline hydrochloride (54 mCi/mg) (17 H) adrenaline hydrochloride (53 mCi/mg) (side chain-12 H) tyramine hydrochloride (121 mCi/mg) (ethylamine 12 H) dopamine hydrochloride (10 mCi/mmol) acetyl (N-methyl-11) choline chloride (11 mCi/mg) (ring ^{14}C) histamine dihydrochloride (>50 mCi/mmol) (^{23}Na) sodium chloride in aqueous solution carrier free (>100 mCi/mg) (^{36}Cl) sodium chloride (>3 mCi/g Cl) (8- ^{14}C) adenosine 5-triphosphate ammonium salt (61 mCi/mmol), (2 H) adenosine 5-triphosphate ammonium salt (1500–30000 mCi/mmol) were obtained from the Radiochemical Centre Amersham.

(Ethyl-1 ^{14}C) β -phenyl ethylamine hydrochloride (313 μ Ci/mg) (^{32}P) disodium phosphate (50–1000 mCi/mmol) were obtained from New England Nuclear Boston.

ABBREVIATIONS

A=adrenaline NA=noradrenaline TA=tyramine
PhEA=phenylethylamine DA=dopamine CA=catecholamine ATP=adenosine-5 triphosphate His=histamine Ach=acetylcholine Na=sodium

RESULTS

1. Dialyzed granule material

Since our previous studies on the binding of biogenic amines (Uvnäs & Åborg 1977) were performed on dialyzed granule material thus depleted not only of CA but also of ATP and inorganic ions our initial experiments in the present series were performed on such material.

Confirming our previous observations the dialyzed granule material was found to retain ^{14}C L-NA and Na. At pH ~7 and within a concentration range up to 10–30 mM in the perfusion fluid the uptake of these cations approached a U_{max} of 400–500 nmol/mg dried granule material (Fig. 3) and the uptake curves fitted the Rothmund Kornfeld equation for weak cation exchangers as previously described by Uvnäs & Åborg (1977). However when the L-NA concentrations were increased above 10–30 mM the L-NA uptake curve was observed to bend more or less steeply upwards indicating the appearance of a second phase of uptake. In the initial experiments which were performed as close to neutral pH as possible the uptake of L-NA could reach unreasonably high values without distinguishable maxima. Since NA has a low solubility and is reported to have a tendency to aggregate at neutral pH we tried to avoid these complications by lowering the pH of the perfusion fluid.

Fig. 3a and 3b illustrate two uptake curves, one for L-NA phosphate (3a) and one for L-NA hydrochloride (3b) both experiments performed at slightly acid pHs as indicated in the figures.

As could be foreseen from our previous observations (Uvnäs & Åborg 1977) there was a considerable distortion and shift to the right of the uptake curve at L-NA concentrations <30 mM due to the leftward shift of the initial phase of uptake to acidification. However using the Rothmund Kornfeld equation

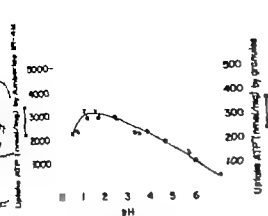


Fig. 9 Influence of pH on uptake of ATP by Amberlite MB3 (anionic exchanger) (—) and by lysed granule material (●—●). Concentration ATP 40 mM.

common biogenic amines tested (PbEA, TA, DA, NA, A, HI, Ach) that were retained above 500 nmol/mg, the U_{max} of pool 1 (Fig. 7). The uptake of LNA and DLA into pool 2 was ~900 and ~400 nmol/mg, respectively.

(b) Uptake of sodium ions

Sodium ions showed a pH sensitive uptake with a U_{max} around 500 nmol/mg (Figs. 6 and 8). In other words, an uptake corresponding to the cation exchanger pool (pool 1). Sodium ions were unable to penetrate into pool 2, even when concentrations of 40–300 mM were used (Figs. 6, 7 and 8).

(c) Uptake of ATP and pH

There was no correlation between the retention of LNA and the anions PO_4^{3-} and Cl^- (Figs. 3, 3b and 5), either when concentrations or pH were varied. When offered as sodium salts the uptake of ATP^{4-} , PO_4^{3-} and Cl^- (in Fig. 8 shown for ATP) increased with increasing acidity indicating the appearance of cationic binding sites—presumably NH_3^+ groups. The conformity between the uptake curves for sodium-ATP by the granule material and by an anionic exchanger (Fig. 4B) is obvious (Fig. 9). However, when perfused through the granule material as Na_2ATP salt, ATP was retained equivalently to NA (Fig. 10), an important phenomenon which will be discussed on page 353.

The possibility that this equivalent uptake of NA and ATP was due to the occurrence of these ions as a complex could be excluded by the fact that the retention curves for Na_2ATP and Na_2ATP (at 50

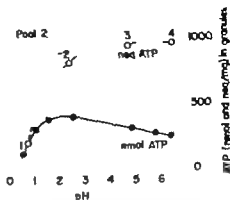


Fig. 10 Influence of pH on the uptake of ATP by lysed granule material with LNA as the cation. LNA 100 mM, ATP 40 mM. Uptake ATP in nmol (●—●) in NaCl (○—○). The alkalies of ATP obtained by titration with HCl denoted by Figs. 1, 2, 3 and 4 respectively.

mM conc. pH < 1 up to 6.5) with HCl were identical.

DISCUSSION

Adrenal medullary granules were lysed by deionized water to disrupt the granule membranes. When such material—with or without previous dialysis—was perfused under slight pressure (10 psi) at $-4^\circ C$ with solutions containing organic (biogenic amines) or inorganic cations these were retained.

The biogenic amines were distributed within two pools. One of the pools (denoted pool 1) was fully charged at amine concentrations > 10 mM up to U_{max} of 400–500 nmol/mg. Pool 1 accepted unselectively biogenic amines (PbEA, TA, DA, NA, A and HI) and inorganic cations (N^+ and Ca^{2+}) and had the characteristics of a cation exchanger. The other pool (pool 2) was selective for NA and A, the only amines among the above mentioned which accumulated towards a higher U_{max} (around 1500–2000 nmol/mg for LNA).

The presence of ATP has been considered essential for the storage of CA in the adrenal medulla, either for ionic binding of CA (Blaschko et al. 1976; Falck et al. 1946) or as a component in aggregate formation (Berners et al. 1971), possibly together with chelating ions (Ca^{2+} and Mg^{2+}). The present observations show that dialyzed granule material depleted of these components still has a considerable ability to retain LNA. Lysed but not dialyzed material has still greater ability to

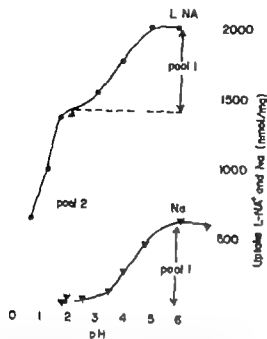


Fig. 6 Influence of pH on uptake of L-NA and of Na by lysed granule material. Concentration of L-NA, ATP and NaCl 700 mM. Note L-NA enters both pool 1 and Na only pool 1. For details see methods.

(a) Uptake of CA and other biogenic amines

Concentration dependence. The non-dialyzed material had a considerably greater ability to retain L-NA than the dialyzed material. Within a concentration range of 10–300 mM L-NA in the perfusate a concentration dependent uptake occurred. The maximal uptake capacity varied somewhat between

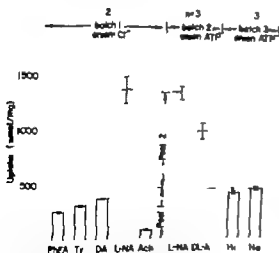


Fig. 7 Total uptake by lysed granule material of various biogenic amines and of Na ions. Concentration throughout 100 mM. Note Only L-NA and DL-A penetrate into pool 2. Uptakes of NA as phosphate and as ATP salt are equal.

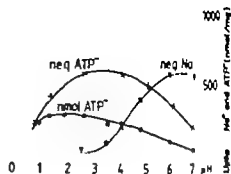


Fig. 8 Influence of pH on uptake of ATP and Na by lysed granule material. Concentration of ATP 700 μ M and of Na 193.5 mM.

batches, usually being between 1400–1600 nmol/mg (Figs 6 and 7).

pH Dependence. In contrast to the initial uptake of L-NA (pool 1) the second phase of uptake was rather independent of pH. It is true that at pH 2.1 the uptake curve for L-NA was shifted somewhat to the right (Fig. 5) but the maximal uptake was still ~1600 nmol/mg. The difference between the maximal uptakes at pH ~6 and 2.1 was ~600 nmol/mg. This figure corresponds fairly well with the size of pool 1 which is abolished at pH 2.

The influence of pH on the uptake of L-NA is seen more in detail in Fig. 6. The uptake curve shows an inflexion point at pH ~2. Above this point the course of the curve reflects the pH sensitivity of the L-NA uptake into pool 1. It should be compared with the corresponding sodium curve below in the same figure. Sodium ions enter only pool 1 as discussed below and shown in Fig. 8. The deduction of pool 1 (~600 nmol/mg for both L-NA and Na) from the total uptake yields pool 2 (~1400 nmol/mg) which as already shown in Fig. 3 is independent of pH above ~2.

Influence of anions. The uptake of L-NA was independent of the accompanying anion as demonstrated for ATP, Cl and PO₄ in Figs 7a, 6b and 7. In the presence of any of these anions the U_{max} for the total uptake reached values around 1400–1600 nmol/mg.

Specific uptake of L-NA and DL-A. As demonstrated previously (Uvnäs & Åborg 1977) pool 1 (the cation exchanger pool) unselectively retains inorganic and organic (biogenic amines) cations with a U_{max} ~400–500 nmol/mg granule material. In contrast pool 2 showed to be specific for L-NA and DL-A which were the only two amines of the 7

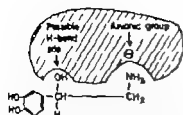


Fig. 2. Hypothetical model of ATP-independent binding of L-NA and L-A to adrenal medullary chromaffin granules.

ing are not essential for the binding mechanism. Presumably then the hydroxyl group in the side chain of NA and A is the essential one behind the specific binding of these amines. The mode of binding of the NA and A can only be speculated upon. To account for the rather pH insensitive but reversible binding of the strongly charged amines one might imagine a binding pocket with an anionic site for the charged amino group—e.g. a COO⁻ group—and a hydrogen bond site for the OH group at the asymmetric carbon atom of the ethyl amine chain (Fig. 1).

We believe that the strongly charged chromogranule molecules—as was shown for the heparin-protonic salt complex—are likely to contain COO⁻—NH⁺ salt linkages forceable by NA and A ions but inaccessible to inorganic cations e.g. K⁺ ions. As pointed out above the ability of NA and A to force the storage pocket of pool is independent of ATP. Although e.g. Cl⁻ and PO₄³⁻ ions do not enter as indicated in Figs 2a, 3b. When NA was offered the granule material as NA-ATP salt ATP was accepted equivalently with NA. The likely explanation is a binding of the acid ATP to ⁺NH₃ groups which become demasked when NA ions break COO⁻—NH⁺ salt bridges and which have an affinity to the phosphate groups of ATP (Fig. 1). Whether in addition to the binding of CA to COO⁻ groups and of ATP to ⁺NH₃ groups also electrostatic interaction occurs between CA and ATP can only be speculated upon. Anyway the postulated equivalent binding of CA and ATP within one and the same 'binding pocket' which behaves like combined anion-cation exchanger should explain the concomitant release of CA and ATP reported by so many authors. The closing of the 'storage pocket' after the two storage components should ensure desired electro-neutrality in the storage machinery.

Unfortunately we had only labelled L-NA and DL-A at our disposal and could therefore not directly study the possible stereospecificity of the CA uptake. However with the same concentrations of the two amines in the perfusate (100 mM) the amount of DL-A retained in pool were ~55% of the amounts of L-NA retained (Fig. 7). Since the two amines seem to have the same affinity to the binding sites of pool (to be published) the observation is compatible with a stereospecific binding of both L-NA and L-A.

The present observations were performed on hyalinated granules at 4°C in order to minimize the uncontrollable influences of active membrane transport processes as well as of enzyme activities. Thus the observed uptake, storage and release phenomena can be characterized as physico-chemical processes non-dependent on metabolic energy.

The functional implication of a granule two-pool storage of NA and A of the kind described above will be discussed in a forthcoming paper in which a cation dependent release of the two CA from medullary granules *in vitro* is described.

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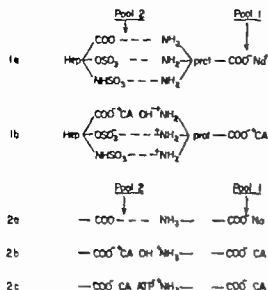


Fig. 11 Schematic drawings illustrating the presumed characters of pool 1 (the cation exchanger pool) and of pool 2 (the storage pool) (1) in a heparin-protamine complex (Uvnäs & Åborg 1976) (2) in lysed medullary granule material. When exposed to sodium ions these ions become attached to free COO^- groups, but are unable to break $\text{COO}^- \text{NH}^+ (\text{NH}_2)$ salt bridges (1a and 2a). When exposed to CA (as hydrochloride or phosphate) CA^+ ions primarily become attached to free COO^- groups but in higher concentrations CA^+ ions force $\text{COO}^- \text{NH}^+ (\text{NH}_2)$ salt bridges open and become attached to the thereby exposed COO^- groups (1b and 2b). Both pool 1 and pool 2 become charged with CA^+ but the anionic Cl^- do not enter. When exposed to CA^+ as ATP salt also the ATP enters pool 2 presumably in ionic linkage with exposed NH_2 groups (c).

store NA corresponding to the normal amounts of CA in the adrenal medulla in vivo.

The mechanism of the NA storage in pool 2 can only be speculated upon. It is evidently independent of ATP, since the uptake (Fig. 7) and the storage capacity as reflected in the U_{max} (Fig. 3a and b) are as high with Cl^- and PO_4^{3-} as with ATP. In contrast to pool 1, pool 2 is rather independent of pH. While the storage ability of pool 1 rapidly deteriorates with increasing acidity and is virtually abolished at pH 3, pool 2 resists acidification down towards pH 2 before it begins to diminish. The concentrations of NA and A required for forcing pool 2 are rather high—above 10–30 mM and U_{max} requires concentrations ~200–300 mM. However, it should be remembered that these concentrations of CA are comparable to those calculated to exist in the medullary granules in vivo (Hillarp 1949).

In its demands for high CA concentrations, its resistance against acidification, its reversibility—the storage of CA increasing with raising and de-

creasing with falling external CA concentrations—and its inaccessibility to sodium ions, pool 2 of the medullary granule material reminds of the second storage pool of a heparin-protamine complex described by Uvnäs & Åborg 1976 (Fig. 11). This salt complex had one cation exchanger pool accessible to both biogenic amines and inorganic cations with the terminal protamine carboxyl groups as the cation binding sites (pool 1) and a second pool (pool 2) accessible to aromatic biogenic amines rather insensitive to acidification and unavailable to low ionic cations like sodium ions. For the opening of pool 2 of the heparin-protamine complex relatively high concentrations of the amines were required (>10–30 mM). From calculations on the binding capacity of the two pools of the complex, it was concluded that when pool 1 was filled, higher concentrations of the amines were able to force open the salt bridge between heparin and protamine, primarily the weaker $\text{COO}^- \text{H}_2\text{N}^+$ bonds and secondly (with even higher concentrations) also the stronger $\text{OSO}_3^- \text{H}_2\text{N}^+$ bonds. The attachment of the amines to the thereby demasked binding sites was reversible and the salt bridges closed as soon as the external amine concentrations were reduced. Only a minor part of the amines were retained in spite of lowering the external amine concentrations, probably due to attachment to the stronger SO_3^- groups.

The medullary granule material has no heparin to form a complex similar to that of the mast cell granules. The proportions of chondroitin sulphate and other sulfomucopolysaccharides are also too small to form protein complexes of sufficient storing capacity (DalPrada et al. 1977). However, the proteins which form the matrix of the adrenal medullary granules are considerably acid with presumably many end terminal α -carboxyls and β - and γ -carboxyls from aspartic and glutamic acid respectively. Titration of granule material for free COO^- groups (figures not shown) revealed more than enough of such groups (~1700 nmol/mg) than required (500–600 nmol/mg) for binding of the CA retained in pool 1, the exchanger pool, but not sufficient for the CA binding in pool 2.

The binding of NA and A in pool 2 is specific, the other aromatic amines tested—PhEA, TA and DA or heterogenic amines like HI and Ach not being able to force this pool open. Especially notable is the inability of the catecholamine DA to do so, indicating that the OH-groups of the catecholamine

In vitro studies on a cation dependent catecholamine release from a two-compartment storage in bovine adrenal medullary granules

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Perfusion of adrenal medullary granules, isolated according to Polaner & Truférø with isotonic cation-containing solutions (NaCl, KCl, LiCl) caused concentration dependent release of CA. The course of the release indicated the existence of two-compartment storage of CA. The major compartment (pool 1) with a U_{max} of ~300 nmol/mg granule dry weight showed the characteristics of a cation exchanger and was assumed to be depleted on exposure to cations e.g. sodium ions, as an ion exchange $Na^+ \rightleftharpoons CA$. The major compartment (pool 2) with a U_{max} of ~1000 nmol/mg stored both CA and ATP. It was assumed to empty as the cation exchanger pool. The total storage capacity of the two compartments ~1500 nmol/mg matched the *in vivo* storage capacity for CA (~1300 nmol/mg according to Hillarp 1960).

Key words: Adrenal medullary granules; storage of catecholamines; noradrenaline; adrenaline; adrenal medulla.

In two preceding papers (Uyváš & Åborg 1977, 1980a) we have described the ability of lysed granule material from bovine adrenal medulla to retain or pass (biogenic amines) and isogenic cations. The observations indicated the existence of storage compartments. One of these denoted pool 1 showed the characteristics of a cation exchanger storing selectively biogenic amines and inorganic cations. The storage capacity of pool 1 amounted to ~400-500 nmol/mg dried granule material. The other compartment, denoted pool 2, showed selectivity for Na^+ and A^+ with a storage capacity for these CA ~1000-1500 nmol/mg granule material. The binding of CA in this pool was tentatively ascribed to the attachment of the amino and hydroxyl groups of the amine side chain to anionic and hydrogen bond sites in the granule matrix (Uyváš & Åborg 1980b). Altogether the total storage capacity for Na^+ and A^+ in the lysed granule material sufficed for the storage of the normally occurring content of the 1 catecholamines (~1300 nmol/mg total granule dry weight according to Palck et al. 1956).

Retained ATP was assumed to be linked—equivalently with CA—to ionized NH groups,

unmasked on the opening by CA of hypothetical $COO^- \cdots H_2N^+$ salt bridges.

In this paper will be described a cation dependent release of NA and A from a two-pool storage system in medullary granules *in vitro* with an attempt to explain the functional interplay between the two pool.

METHODS

Bovine adrenal medullary granules were prepared by multiple filtration of homogenized adrenal medulla according to Polaner & Truférø (1967), as described by Uyváš & Åborg (1980). The granules were resuspended in 0.32 M sucrose and 1 ml of this suspension put on 25 mm Ø filter pore size 0.01 µm Sartorius SM 11311 m Sartorius filter apparatus Type SM 16223. The sucrose solution was removed under an N_2 gas pressure of 700 KPa. A second filtrate of the same characteristics as above was then removed and the granule layer and tightly sealed with a rubber packing.

7 ml of isotonic NaCl or other isotonic solutions (usually buffered with 10% $Na_2HPO_4 + H_2SO_4$ to pH 7.4) were passed through under an N_2 pressure of 700 KPa. The effluent were collected in preweighed plastic tubes (5.5 × 1.1 cm) with stoppers in 0.25–0.5 ml samples. The

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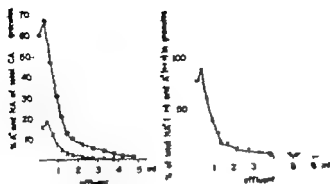


Fig. 3. (a) Constant release of A and NA from granules perfused with isotonic (163 mM) NaCl expressed as (—) per cent of total CA, (---) per cent A and NA of total A and NA respectively.

express the bound CA according to Scatchard (Fig. 5). The plot may suggest a two-pool system with a maximal storage capacity of ~ 500 and ~ 1000 nmol/mg respectively. A reciprocal plotting of the CA concentrations in the perfusion fluid vs. the corresponding concentrations in the granules may likewise be interpreted as indicating a two-pool system with maximal capacity values around 500 and 1000 nmol/mg respectively (Fig. 5d). Plotting the effluent values according to the Rothman-Korfeld equation for weak cation exchangers showed that only the values corresponding to the later part of the effluent curve—points 9–15—fit this equation while the earlier effluent values do not (Fig. 5e).

The curves plotted above may be taken to

suggest that the granule CA store comprises compartments, one the minor one with the characteristics of a weak cation exchanger (the other the larger one of so far undefined nature).

An attempt to envisage the interplay between the two hypothetical CA storage pools instrumental at sodium induced CA release is seen in Fig. 6. Using values from curves 5a–f the curves in Fig. 6 were constructed. By deduction of the curve for pool 1 (the exchanger pool) from the curve for the total store the curve for pool 2 is visualized. Fig. 6 also illustrates the inverse relationship between the CA release and the Na uptake by ion exchange in pool 1 with the constant sum of intragranular CA and Na as demonstrated above (Fig. 4). The curves will be further commented upon under Discussion.

7

NA induced inhibition of CA release

The slight leakage of CA observed on perfusion of the granules with isotonic sucrose was inhibited by the addition of L-NA to the perfusion fluid. In the presence of 10 mM L-NA the net loss of CA levelled off at an intragranule concentration ~ 1050 nmol/mg (Fig. 7). Noteworthy is the agreement between this experimentally obtained value and the corresponding value that could be predicted from Fig. 6 (point a). The presence of 10 mM L-NA also limited the CA released induced by perfusion of the granules with isotonic sodium chloride solution. In fact the net release of CA was arrested at an intragranule concentration ~ 550 nmol/mg. Also this value is pretty close to the one predictable from Fig. 6 (point b) at this extragranule CA concentration.

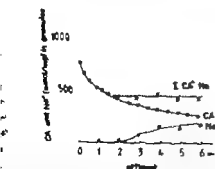


Fig. 4. Release of CA and uptake of Na in granules perfused with isotonic (163 mM) NaCl buffered with 10% sodium phosphate buffer to pH 7.4. Release of CA (O—O); uptake of Na (●—●). Sum of CA release and Na uptake by the granules (—). Note: CA and Na constant at ~ 500 nmol/mg corresponding to the U_{max} of pool 1, the cation exchanger pool.

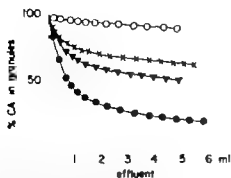


Fig. 1 Release of CA from granules perfused with isotonic solutions containing deionized sucrose (O—O), sucrose + 40 mM Na (X—X), sucrose + 80 mM Na (▽—▽) and 163 mM Na (●—●). All the NaCl containing solutions buffered to pH 7.4 with 10% sodium phosphate buffer.

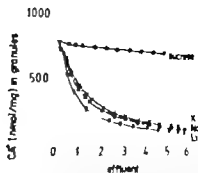


Fig. 2 Release of CA from granules perfused with isotonic solution of sucrose (O—O), potassium chloride (X—X), sodium chloride (●—●) and lithium chloride (▽—▽). All cation containing solutions buffered to pH 7.4 with phosphate buffer of the respective cation.

exact volumes were obtained by weighing $\pm 5 \mu\text{l}$ from each sample were mixed with 5 ml 0.1 M HCl containing 0.005 M H_2SO_4 . CA was determined spectrofluorimetrically (fluorescence 315 nm excitation, 385 nm).

0.1 ml from each sample was used for protein determination according to Lowry et al. (1951).

ABBREVIATIONS

CA=catecholamine; A=adrenaline; NA=noradrenaline; ATP=adenosine 5' triphosphate.

RESULTS

Cation induced CA release

Perfusion with isotonic deionized and unbuffered sucrose solution resulted in a slight release of CA (Fig. 1). However, when the sucrose was exchanged for sodium chloride the release was accelerated and enhanced the more so the higher the sodium content (Fig. 1). Isotonic solutions of K, Na and Li-chloride yielded very similar release curves (Fig. 2). The concomitant outflow of PCA precipitable protein amounted to less than 1% of the total granule content of such protein, indicating negligible losses of granule matrix protein.

The release included both A and NA with practically identical release curves for the two amines on exposure of the granules to isotonic NaCl solution (Fig. 3a and 3b).

Entrance of sodium ions into granules

Concomitant recordings were made of the appearance of CA in the perfusion fluid and the entrance of Na ions into the granules. The granules were perfused with isotonic NaCl solution. No detectable

accumulation of sodium (^{22}Na) occurred in the granules until their CA content had declined below 500 nmol/mg. From then on a continuous accumulation of Na occurred concomitantly with a continued depletion of the CA content (Fig. 4). A noticeable fact is that the sum of the equivalents of Na which entered the granules and those of the CA that left the granules remained constant at ~ 900 nmol/mg. It is further noticeable that the entrance of Na into the granules began first when the intragranular content of CA had fallen to ~ 900 nmol/mg, just the CA level which according to the calculations below (Fig. 5) represents the U_{max} of pool 1, the cation exchanger pool. The observations thus agree with an ion exchange between Na and CA ion in pool 1.

Evidence for a two-compartment storage of CA

Our previous studies on the CA storing ability of lysed granule material had suggested the existence of a two-pool storage of CA in that material. An analysis of the release curves for CA from perfused granules induced by the cations Na, K and Li suggested a similar two-pool system in the intragranule storage of CA. The release curves in Figs. 5a and 5b show the release of CA (nmol/mg) into the effluent and the remaining CA (nmol/mg) in the granules respectively on perfusion of the granules with 163 mM NaCl. Out of totally 1097 nmol/mg in the granules, 9.4 nmol/mg were released during the perfusion.

Assuming some kind of competitive binding of CA to storage sites in the two pools, the ratio between granule bound and free CA was plotted

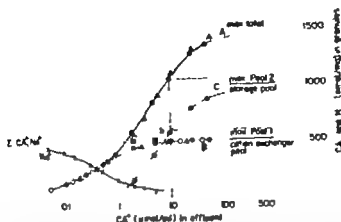


Fig. 6. Constructed curve to illustrate the envisaged interaction within two-pool system for the storage and release of CA in adrenal medullary granules. Curves: A. Total CA store. Δ — Δ , from Scattergood plot (Fig. 5); \bullet — \bullet , from Lineweaver-Burk plot (Fig. 5d); Curves B. CA in pool 1, the exchanger pool. \circ — \circ , from Rothmund-Kornfeld (Fig. 5e); Δ — Δ , from Scattergood plot (Fig. 5c); \bullet — \bullet , from Lineweaver-Burk plot (Fig. 5d); Curves C. CA in pool 2, storage pool. Curves D. CA in pool 1 deduced from Curve A (total CA store). — Sodium content in pool 1 (from Rothmund-Kornfeld) (Fig. 5e). \square — \square Σ CA + NA in pool 1.

free intact granules yield a picture of a two-pool storage system very similar to that one obtained from our previous uptake studies on lysed granule material (Uvåas & Åborg 1980, Fig. 3). The intra-granule localization, the chemical construction and the inter- and intramolecular arrangement of the two pools are unknown, but an attempt to interpret our observations in functional terms and to envisage a possible interplay between the pools is schematically indicated in Fig. 8. This picture should not be seen as an indication of the partial

arrangement of the two pools only as an attempt to visualize the role of pool 1 as a transfer pool for the release of pool 2, the storage pool in our hypothetical scheme. However, the two pools seem to be localized to the vesicle matrix, since no appreciable CA storing capacity was observed in the granule membrane fraction precipitated by differential centrifugation ($100\,000 \times g$, 30 min). The storing capacity remained in the supernatant.

According to our hypothesis the one of the CA containing pools by us denoted pool 1 has the properties of a cation exchanger. When this pool is exposed to biogenic amines (in vivo presumably mainly A and NA) or inorganic cation (in vitro presumably mainly sodium ions) these positively charged ions will be unselectively retained according to the prevailing concentrations and to their affinities to the anionic binding sites in the pool, probably protein carboxyls. The storage ability of pool 1 is rather sensitive to acidification (see e.g. Fig. 5 in Uvåas & Åborg 1980) and might therefore depend on the β and γ carboxyls of aspartic and glutamic acids which have pK values ~ 3.7 and 4.3 respectively. Pool 1 has a maximal storage capacity ~ 400 nmol/mg granule dry weight at external cation concentration ~ 10 mM at neutral pH.

The other of the two CA containing pools by us denoted pool 2, is in contrast to pool 1 specific—to judge from our observations on lysed granule ma-

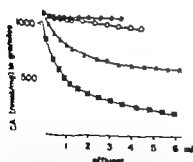


Fig. 7. Laboratory effects of 10 mM NA (ammonium ATP) on CA release. Granules perfused with isotonic sucrose (O—O), isotonic sucrose + 10 mM NA (●—●), isotonic NaCl (163 mM) (□—□), isotonic NaCl + 10 mM NA (ammonium ATP) (■—■). pH ~ 6.2 .

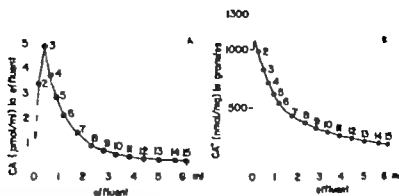


Fig. 5a Release of CA (μmol/ml effluent) on perfusion of granules with 163 mM NaCl buffered with 10% sodium phosphate buffer to pH 7.4

Fig. 5b CA (nmol/mg) in granules perfused with isotonic (163 mM NaCl) buffered with 10% sodium phosphate buffer to pH 7.4

DISCUSSION

A two-compartment storage of medullary CA

Supported by the present studies on the release of CA from isolated granules and the previous studies on the uptake of CA by lysed granule material (Uvnäs & Åberg 1979) we propose a two-pool storage

of CA in the adrenal medullary granules. Fig. 6 shows a graphical construction of these two pools based on the cation induced CA release observed in the present studies. These values for the CA release

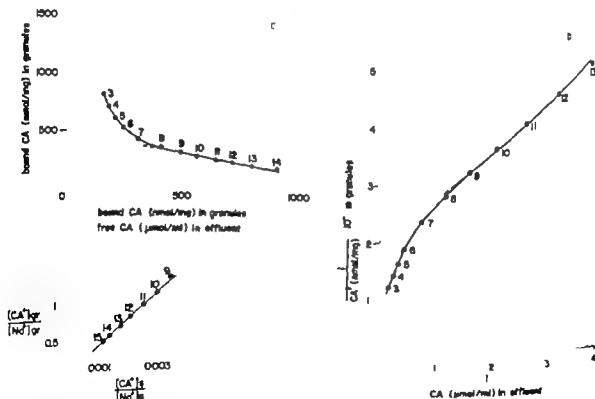


Fig. 5c Scatchard plot derived from the release curves in Fig. 5a and 5b

Fig. 5d Lineweaver-Burk plot derived from Fig. 5a and 5b

Fig. 5e Logarithmic plotting of the ratios between the concentration of CA and Na⁺ in the granules vs. the corresponding concentrations in the effluent on perfusion of the granule with 163 mM NaCl buffered to pH 7.4 with 10% sodium phosphate buffer. Values obtained from Fig. 5a and 5b. The values follow a straight line i.e. fulfil the Rothmund-Kornfeld equation for a weak cation exchanger. U_{max} 400 nmol/mg

When medullary granules are perfused with inorganic cations, i.e. sodium ions, such ions will penetrate into the granules. Na⁺ ions are unable to enter pool 1, which is specific for L-Asp and L-Glu (as shown previously by Uvnäs & Åborg 1979) but will compete with CA ions at the ionic sites of pool 2. As CA ions are released and pass out through the granule membrane (arrow 1 and 2 in Fig. 8). However, as long as pool 2 is filled pool 1 will be under permanent high CA ionic pressure, the more so the more pool 2 is filled. The Na⁺ ions which enter pool 2 will therefore immediately be replaced by CA ions from pool 2 (arrows 3 and 4 in Fig. 8). If completely filled the CA ionic pressure will correspond to a CA concentration of 300–300 mM. A concentration more than 10-fold exceeds the concentration required for the saturation of pool 2 (10 mM). No detectable amounts of Na⁺ ions will therefore be able to accumulate in pool 2. However, as pool 2 is depleted the CA ionic pressure on pool 2 declines, Na⁺ ions can more obviously compete for the ionic sites of pool 2 and the CA release is increasingly accompanied by the accumulation of Na⁺ ions in this pool as illustrated in Fig. 6. The concentrations of NaCl required to release CA might seem high but are explained by the fact that the affinity of CA ions for the ionic sites of the ion exchanger pool is >10 times higher than the affinity of Na⁺ ions for the same sites (Uvnäs & Åborg 1977).

The idea of a two-compartment store of CA in the adrenal medulla is not new. Hillarp (1960) suggested the existence of two storage pools to explain the observed equivalent excess of CA over ATP. Similar arguments for a two-pool system were brought forward by Stjärne (1964), Taupner & Hasselbach (1966) and others.

In the present two-pool hypothesis the larger compartment (pool 2) should correspond to the proton-ATP-CA complex postulated by various authors. Even according to our observations ATP is essential for the binding of CA in pool 2 as demonstrated on lysed granule material. It might serve as a stabilizing factor by its possible electrostatic attraction to CA (Fig. 8). The indicated intramolecular arrangement between CA and ATP should also explain the concomitant release of CA and ATP. When the CA ions move from pool 2 to pool 1 the neutrality is maintained by the simultaneous loosening of ATP. The COO⁻ and H₂N⁺ ions of the storage sites reattach to each other and

neutrality becomes restored by the closing of the "storage pocket".

The smaller compartment (pool 1)—in our scheme the cation exchanger pool—should correspond to the previously suggested ATP-free CA pool.

The existence of a third pool of free diffusible CA has been lively discussed (Euler 1956, Stjärne 1964). In fact in order to uphold the ionic balance and the ion flux between pool 1 and pool 2 in our scheme as well as between pool 1 (the release pool) and the extragranule space minute fractions of diffusible or free CA ions have to be imagined. Whether these diffusible minute fractions of CA ions should be denoted pools or not seems to us to be more of a semantic question.

In this context the observed leakage of CA from granules perfused with isotonic sucrose might deserve some comments. Since the granule membranes at 0°C are permeable to CA ions a leakage of CA will be an inevitable consequence of the existence of free CA pools as discussed above. This observed leakage of CA seemed to be contradictory to previous reports that granules suspended in isotonic media maintained their CA content for days (Falck et al 1956 and others). However, we found that iterated resuspensions of batches of granules in fresh isotonic sucrose or salt solution led to a stepwise reduction of the granule CA content. After each resuspension the CA content in the granules assumed a new lower level but remained then constant for hours, indicating that the retention of CA by the granules depended on the equilibrium formed between intragranule and in the suspension fluid accumulated CA. In our perfusion system such an equilibrium between intra- and extragranule CA concentrations will not occur due to the continuous removal of the released CA. However, as predicted from the above reasoning the net loss of CA from our perfused granules could be effectively counteracted by adding NA in appropriate concentrations to the perfusion fluid. In other words, the observed ability of isolated medullary granules to retain CA at 4°C is not due to any unique properties of the granule membrane. It is the simple result of a physico-chemical balance between intra- and extragranule CA.

The experiments described in the present article have all been performed on isolated medullary granules at 4°C. Consequently membrane transport processes have been "paralysed" and enzymatic processes practically abolished. The observed in-

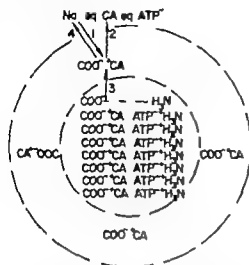


Fig. 8 Schematic drawing of the functional interplay between cation exchanger pool (pool 1) and storage pool (pool 2). For explanation see text p. 361. The picture should not be taken to suggest the spatial organisation of the two pools in the granule.

terial possibly stereospecific—for L A and L NA. The maximal storage capacity of pool 1 is ~1000 nmol/mg. For its filling pool 2 requires higher external CA concentrations than pool 1 for maximal uptake ~200–300 mM. Pool 2 is reversible: the number of L A and L NA storing "pockets" increases with raising and decreasing with falling CA concentrations. Since as already mentioned above pool 2 is specific for L A and L NA (DA is not retained in this pool only in the unselective pool 1) and the hydroxyl group in the catecholamine side chain seems to be essential for the storage in pool 2 we have suggested (Uvnäs & Åborg 1979, Fig. 9) that L A and L NA are held in pool 2 by the combination of ionic binding of the NH₃ groups in the amine side chain to protein carboxyls and of hydrogen bonds between the hydroxyl group of the side chain and hydrogen binding sites of the granule protein. Since pool 2 is rather insensitive to acidification—the storage mechanism remains seemingly intact down towards pH ~ 4—terminal protein carboxyls with their pK_a ~ 2 might be involved as binding sites.

The sum of the storage capacity of pool 1 (~500 nmol/mg) and pool 2 (~1000 nmol/mg) compares well with the value for the total content of CA in the bovine adrenal medulla (~1300 nmol/mg) reported by Hillarp et al. 1955.

In the present experiments ATP was observed to be released from the granules concomitantly and equivalently with CA (unpublished). This observa-

tion is in accordance with a storage of ATP in pool 2 as proposed by Uvnäs & Åborg 1979, and schematically illustrated in Fig. 8. According to their proposal ATP is ionically linked to NH₃ groups which become unmasked on the opening by CA of hypothetical COO⁻ --- NH⁺ salt bridges in the protein matrix of the chromaffin granules. CA is ionically linked to COO⁻ groups in the same storage pocket. This simple mechanism of CA and ATP binding to ionic sites of opposite charge as a reversible storage pocket explains the equivalent storage and release of the two components as is often reported in the literature. By this reversible binding mechanism the desirable electro-neutrality of the storage mechanism is automatically secured. Additional stabilizing intermolecular attractive forces between CA and ATP as suggested by many authors to form an important part of the storage mechanism is easily compatible with our storage hypothesis. However as pointed out in our previous paper (Uvnäs & Åborg 1979) the presence of ATP is not a prerequisite for the storage of CA. Only A and NA are able to force the specific storage sites of pool 2 and ATP will enter as ion of opposite charge according to supply. Since Cl⁻ and PO₄³⁻ ions do not enter the storage sites these sites will probably in the lack of ATP be supplied with OH⁻ ions. Excess of ATP on the other hand will not lead to an increased storage of ATP in pool 2, as shown by studies with sodium-ATP. ATP ions alone are not able to enter pool 2.

Functional interplay between pool 1 and pool 2

As well known at 0°C the membrane of isolated medullary granules is permeable to smaller ions like CA, ATP, Na⁺ etc. but impermeable to macromolecules (for ref. see Stjärne 1974). We would therefore like to see the release of CA occurring in the present experiments on perfusion of the medullary granules with isotonic NaCl solution as the result of a cation exchange between CA and Na⁺ ions in pool 1, the exchanger pool. Such an interpretation might at first sight seem incompatible with the fact that no accumulation of Na⁺ ions is observed to occur in the granules until they have lost the major part of their CA. However, guided by Figs 7 and 8 we want to envisage the possible interplay between the two CA pools with a cation exchange over pool 1 as the essential step. Our working hypothesis is as follows.

Possible role of nerve impulse induced sodium ion flux in a proposed multivesicular fractional release of adrenaline and noradrenaline from the chromaffin cell

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Based on our observations concerning a two-compartment storage of CA in the adrenal medulla and calcium channel dependent release of CA from perfused chromaffin granules *in vitro* and encouraged by recent reports from other laboratories about the importance of sodium ions for the CA release from the adrenal gland we propose a modification of the current quantal theory of CA secretion. Instead of secretion of quanta each quantum corresponding to the content of one vesicle, we argue a concentrated fractional release of CA from multiple vesicles adjacent to the chromaffin cell membrane. The CA secretion should be the result of calcium exchange across the contact area between the plasma membrane and the granule membrane during the period of depolarization caused by the nerve impulse. The size of the released quanta should be determined by the nerve impulse induced sodium ion flux and the number of such ions which reach the CA binding ionic sites in the calcium exchanger pool (the release pool) of the granules.

Key words: Adrenaline, noradrenaline, release, chromaffin cell, adrenal medulla.

According to the doctrine of the day the secretion of adrenal catecholamines (CA) is the result of an exocytosis of CA containing vesicles with a total liberation of the affected vesicles not only of their content but also concomitantly of all other soluble components like ATP, chromogranins, dopamine, β -hydroxytyramine etc. This all or none exocytotic response implies that the CA secretion is quantal with the content of a vesicle as the smallest quantum to be secreted. The vesicles are assumed to be in random motion (Brownian motion) and to collide randomly with the plasma membrane of the chromaffin cell. On nervous and humoral stimulation active sites are assumed to occur in the chromaffin cell membrane and vesicles which hit such an activated site become attached to the plasma membrane. The granule and plasma membranes fuse and an opening is formed through which the vesicle content is evacuated into the extracellular space (Fig. 1 and 1b).

The quantal theory with the CA content of one vesicle as the smallest releasable quantum further implies that this quantum is the smallest amount to

be released by a nerve impulse. A chromaffin cell from cat adrenal is claimed to contain 13000 CA vesicles (Auerbach & Viveros 1970). The amount of CA released by one splanchnic nerve stimulus is calculated to 3.5×10^{-10} (Ceflander 1954) and to 3×10^{-10} (Kirschner & Viveros 1970) of the total CA content of the gland. This amount of CA corresponds to $\sim 1/4$ of a vesicle content per chromaffin cell. The extreme consequence of the quantal theory should then be that a splanchnic nerve impulse volley should cause at random the secretion of one single CA vesicle (out of 13000) in every third chromaffin cell.

Let us assume then that under regular *in vivo* conditions the impulse frequency in the splanchnic nerve is $\sim 1/s$ and under stress conditions $\sim 10/s$. In case no resynthesis and no recapture of the secreted CA occurred the total adrenal CA store should be depleted within 10 and 2 h respectively. In most experimental studies on nervous release of CA stimulation frequencies of 20-30 Hz have been used. The chromaffin cells should under these conditions be depleted of all their CA content within half an hour.

duction and inhibition of CA release may therefore be considered as due to non-energy requiring physico-chemical processes: simple cation exchange playing an essential role. The absence of diffusion barriers in the granule membrane renders it difficult to translate our present *in vitro* findings to *in vivo* conditions. However in a forthcoming paper we will present arguments in favour of a nerve induced cation exchange instrumental in the release of CA from the adrenal chromaffin cells.

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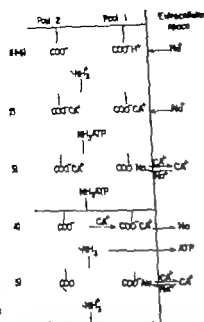


Fig. 3. Schematic drawing of the proposed interplay between release pool and storage pool in the chromaffin cell. (1) The two pools are empty. The COO⁻ groups in pool 1 are occupied by H⁺ ions. The COO⁻ groups in pool 2 are occupied by Na⁺ ions. (2) The two pools are filled with CA. In pool 1 the COO⁻ groups are occupied by CA. In pool 2 the COO⁻ groups are occupied by CA. (3) Due to nerve stimulation, the influx of sodium ions such ions reach the COO⁻ groups of the cation exchanger pool and the CA is released by Na⁺ ions and released. (4) Due to the high concentration of CA in the storage pool CA from this pool immediately moves towards the COO⁻ groups of the release pool. Na⁺ ions will not accumulate in this pool until the CA present from pool 1 is sufficiently reduced. The membrane potential of pool 2 is established by the outward release of ATP and the COO⁻ NH₃ salt bridge is closed. (5) With depletion of pool 1 sodium ions will increasingly accumulate in this pool.

Our argumentation for a cation exchange dependent CA release from a two- (or three) pool system in the chromaffin cell follows below.

The importance of sodium ion for CA release

The entrance of sodium ions into chromaffin cell is essential for CA release. Recent reports suggest an essential role for Na⁺ ions in the CA release pro-

cess. According to Banks (1967) and Banks et al (1968) perfusion of bovine adrenals with sodium-free Tyrode solution abolished or severely reduced the secretory response to carbamylcholine and to 70 mM KCl. Procedures assumed to increase intracellular sodium such as perfusion with potassium-free or ouabain-containing Tyrode solution increased the basal CA secretion. Veratridine induced CA secretion from perfused guinea pig adrenals was blocked by tetrodotoxin (TTX) presumably due to blockade of sodium channels opened by veratridine (Ito, Nakazako & Ohga 1978). Knight & Whitaker (1978) reported that substitution of choline for sodium in the perfusion medium or addition of TTX abolished the CA release in isolated bovine medullary cells exposed to veratridine.

The functional importance of sodium ions in the CA release process indicated by the above cited observations has led most authors to suggest that the opening of sodium channels and (or) the entrance of sodium ions should facilitate the entrance of Ca²⁺ ions, a step which is commonly accepted as essential for the initiation of the release process. Our observations on the cation induced CA release (by calcium free isotonic NaCl solutions) from isolated chromaffin granules (Uvnäs & Åberg 1980) might rather indicate that the entrance of Na⁺ by Ca²⁺ ions are parallel phenomena, sodium ions being responsible for cation exchange Na⁺ ↔ CA at ionic sites in granules which—possibly after the mediation of Ca²⁺ ions—have become attached to the chromaffin cell membrane.

Ca²⁺ quantal adrenal CA secretion be explained solely as intragranular cation exchange between CA and Na⁺ ions. The electrophysiological characteristics of the chromaffin cell membrane and the nerve membrane have been supposed to be widely different. The potential difference over the medullary cell membrane should be too low to allow any propagated action potentials, only sufficient for local minimal depolarization (Kahn & Douglas 1967). However, recent observations on cultured medullary cells from rat (Brandt et al 1978) and from gerbil and adult human adrenal medullary cell (Blaes, Dichter & Aleschler 1978) convincingly demonstrated resting potentials exceeding 50 mV, as well as overshooting action potentials similar to those generated by sympathetic neurones. The action potentials disappeared either upon removal of external sodium ions or addition of TTX. The conclusion was drawn that the action potentials in the chro-

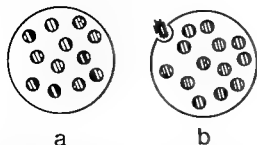


Fig. 1 According to the current view CA secretion from the chromaffin cell is quantal: the CA content of a vesicle being the smallest releasable quantum. Together with CA all other soluble components of the vesicle are released. (a) Resting chromaffin cell. (b) Nerve impulse induced total discharge of the content of one CA vesicle.

As well known the chromaffin cell has a considerable capacity to resynthesize CA. However, a secretory process that requires the rapid resynthesis of not only the hormone or transmitter. In this case CA, but also of all other storage components including matrix proteins and various synthesizing enzymes seems to be a rather wasteful machinery.

We have in a series of papers reported on observations concerning a two-compartment storage of CA in the adrenal medullary granules (Uvnäs & Åborg 1977, 1980a and b). The larger of these compartments, capable of storing two thirds of the adrenal CA was specific—probably stereospecific—for A and NA. The smaller of the compartments showed the properties of a weak cation exchanger, unselectively accepting inorganic and organic (biogenic amine) cations. Subsequent studies on a cation induced CA release from adrenal medullary granules *in vitro* suggested to us that this CA release was the result of a cation exchange (Na^+ CA⁻ ions) in the cation exchanger pool—by us denoted the release pool—and that this release pool also served as a transfer pool for the depletion of the storage pool (see Fig. 8 Uvnäs & Åborg 1980b).

On the basis of the above-mentioned studies on the storage and release of CA and supported by recent observations in other laboratories on the significance of sodium ions for the release of adrenal CA we propose a hypothesis according to which the nervously (and possibly also the humorally) evoked secretion of adrenal CA is quantal although not with the total content of single vesicles as the smallest releasable quanta but with a concomitant fractional release of CA from multiple vesicles. The size of the released quanta of CA is assumed to depend on a nerve impulse induced sodium ion flux

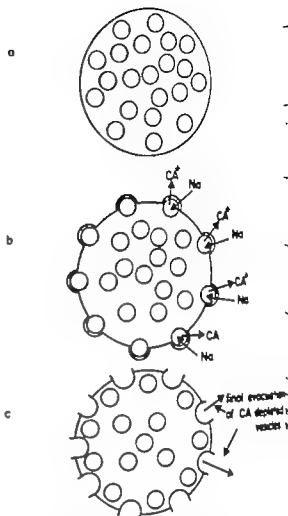


Fig. 2 Proposed hypothesis of a quantal but multifactorial fractional release of CA from the adrenal chromaffin cell. The size of the quantum of CA secreted depends on the nerve impulse induced sodium ion influx across the contact area between the chromaffin cell membrane and the vesicle. (a) Resting chromaffin cell with numerous CA vesicles adjacent to the plasma membrane. (b) Nerve impulse induced depolarization of chromaffin cell membrane with attachment of vesicles to the membrane. Subsequent flux through open sodium channels results in CA release via cation exchange between Na^+ and CA ions in granule cation exchanger (release) pool. The fractional release allows a continuous refilling of the CA store by resynthesis. (c) Total evacuation of soluble vesicle components, an infrequent phenomenon under regular *in vivo* conditions and occurring only as an ultimate step in order to dispose of worn out vesicles.

and subsequent cation exchange (Na^+ CA⁻) across the contact area between the plasma membrane of the chromaffin cell and CA vesicles attached to this membrane. The total evacuation of vesicles is considered as an ultimate under normal *in vivo* conditions rather infrequently occurring disposal of storage and building materials from worn out vesicles (Fig. 2).

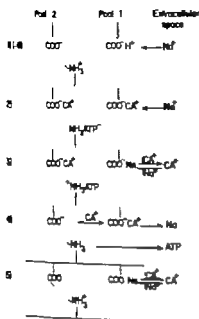


Fig. 3. Schematic drawing of the proposed interplay between release pool and storage pool in the chromaffin granule (denoted pool 1 and pool 2 respectively). Pool 1 is cation exchanger pool in which COO⁻ groups selectively accept biogenic amines and inorganic cations. Pool 2 is anion exchanger pool in which COO⁻ groups are selective for L-A and L-NA and its storage function is dependent on the ability of these two catecholamines to form COO⁻—NH₃ salt bridges. Sodium ions are not able to enter this pool (for further details see Ulfhake & Åberg, 1974b). (1) The two pools are empty. The COO⁻ groups in pool 1 are occupied by H⁺ ions. The COO⁻—NH₃ salt bridges are closed. (2) The two pools are filled with CA. In pool 1 the COO⁻ groups are occupied by CA and in pool 2 denatured COO⁻ and NH₃ groups are occupied by CA and ATP respectively. (3) Due to nerve impulse induced influx of sodium ions such ions reach COO⁻ groups of the cation exchanger pool and the CA is released by Na⁺ ions and released. (4) Due to the high concentration of CA in the storage pool CA from this pool immediately moves towards the COO⁻ groups of pool 1. Na⁺ ions will not accumulate in this pool until the pressure from pool 1 is sufficiently reduced. The electrical permeability of pool 2 is reestablished by dependent release of ATP and the COO⁻—NH₃ salt bridge is closed. (5) With depletion of pool 1 sodium ions increasingly accumulate in this pool.

Our argumentation for a cation exchange dependent CA release from a two- (or three) pool system in the chromaffin cell follows below

Importance of sodium ions for CA release

Entrance of sodium ions into chromaffin cells is essential for CA release. Recent reports suggest an essential role for Na⁺ ions in the CA release process.

According to Banks (1967) and Banks et al (1968) perfusion of bovine adrenals with sodium-free Tyrode solution abolished or severely reduced the secretory response to carbamylcholine and to 70 mM KCl. Procedures assumed to increase intracellular sodium such as perfusion with potassium-free or ouabain-containing Tyrode solution increased the basal CA secretion. Veratridine induced CA secretion from perfused guinea pig adrenals was blocked by tetrodotoxin (TTX) presumably due to blockade of sodium channels opened by veratridine (Ito Nakazako & Ohga 1978). Knight & Whittaker (1978) reported that substitution of choline for sodium in the perfusion medium or addition of TTX abolished the CA release in isolated bovine medullary cell exposed to veratridine.

The functional importance of sodium ions in the CA release process indicated by the above cited observations has led most authors to suggest that the opening of sodium channels and (or) the entrance of sodium ions should facilitate the entrance of Ca²⁺ ions a step which is commonly accepted as essential for the initiation of the release process. Our observations on the cation induced CA release (by calcium free isotonic NaCl solutions) from isolated chromaffin granules (Ulfhake & Åberg 1980) might rather indicate that the entrance of Na⁺ and Ca²⁺ ions are parallel phenomena, sodium ions being responsible for cation exchange Na⁺ ↔ CA at ionic sites in granules which—possibly under the mediation of Ca²⁺ ions—have become attached to the chromaffin cell membrane.

Can quantal adrenal CA secretion be explained as due to 1:1 granule action exchange between CA and Na⁺ ions? The electrophysiological characteristics of the chromaffin cell membrane and the nerve membrane have been supposed to be widely different. The potential difference over the medullary cell membrane should be too low to allow any propagated action potentials, only sufficing for local minimal depolarization (Kann & Douglas 1967). However recent observations on cultured medullary cell from rat (Brandt et al 1978) and from gerbil and adult human adrenal medullary cells (Bales Dichter & Benschler 1978) convincingly demonstrated resting potentials exceeding 50 mV as well as overshooting action potentials similar to those generated by sympathetic neurones. The action potential disappeared either upon removal of external sodium ions or addition of TTX. The conclusion was drawn that the action potentials in the chro-

chromaffin cell membrane were due mainly to increased Na^+ ion permeability.

During the millisecond of depolarization there will presumably be a free inward flux of Na^+ ions across the chromaffin cell membrane. Assuming that vesicles adjacent to the chromaffin cell membrane are attracted and attached to this membrane (due to the action of Ca^{2+} ions entering concomitantly with Na^+ ions²) the inwardly flowing Na^+ ions will reach the membranes of the peripherally located granules and the postulated ion exchange $\text{Na}^+ \rightleftharpoons \text{CA}^+$ ions may occur. Whether the prevailing permeability of the granule membrane for CA^+ and Na^+ ions suffice for the required ion passage or if the formation of semipermeable sites or opening of pores are needed can at present only be speculated upon. However, some reflexions as to the size of the ion flux that might be induced by a synaptic nerve impulse across the contact surface between the chromaffin cell and the granule membranes might be possible from available data on sodium ion fluxes across other membranes.

Hodgkin (1963) estimated the sodium ion flux in an unmyelinated giant nerve axon to about 70 000 and at a node of a myelinated nerve axon to about 300 000 $\text{ions}/\mu\text{m}^2/\text{impulse}$. Caterall (1979) calculated the density of sodium channels in synaptosomes from rat brain to 25–50 $\text{channels}/\mu\text{m}^2$ and the sodium ion flux/channel to 1×10^6 ions/min corresponding to a flux of 42 000–84 000 $\text{ions}/\mu\text{m}^2/\text{ms}$.

With an estimated diameter of a chromaffin vesicle of 2.5×10^{-6} μm the surface of the vesicle (assumed to be spherical) will be

$$4 \pi \left(\frac{2.5}{2}\right)^2 10^{-6} \mu\text{m}^2 = 19.6 \times 10^{-12} \mu\text{m}^2$$

(approx. 2×10^{-11} μm^2). If we assume that the figures for the density of sodium channels and the size of the sodium flux in the chromaffin cell membrane approximate those of the synaptosomal membranes of the rat brain the sodium ion flux across an area of the chromaffin cell membrane of corresponding size should reach values around $7 \times 10^{-4} \times 42\,500$ – $85\,000$ = 8500–17 000 ions/ms . Calculated according to Hodgkin's figures for the unmyelinated giant axon ($70\,000/\mu\text{m}^2/\text{ms}$) the corresponding ion flux across the chromaffin cell membrane should be $20\,000 \times 7 \times 10^{-11} = 4\,000$ ions/ms .

Kirchner and Viveros have estimated the number of CA vesicles in a chromaffin cell to 13 000 and the CA content per vesicle to 7.2×10^{-16} g corresponding to 2.4×10^4 molecules. Since the CA release per

nerve impulse was calculated to about 1/3 of the CA content of one vesicle (3) i.e. $\sim 10^4$ molecules, a CA release due to cation exchange should require an equivalent amount of sodium ions to occupy the CA binding sites. If during the exocytotic process the contact area between a vesicle and the chromaffin cell corresponded to the whole vesicle surface and the sodium ion flux amounted to 10 000 ions/ms (see above) a minimum of 100 vesicles had to be engaged in the nerve impulse induced CA release, 1/3 of the vesicle content being released by each impulse. Probably the whole surface of the vesicle is not in intimate contact with the chromaffin cell membrane but on the other hand the number of vesicles concomitantly involved in the CA secretion might be considerably higher. In fact with a mean chromaffin cell diameter of 1×10^{-6} cm and a mean vesicle diameter of 2.5×10^{-6} cm the inner surface of the chromaffin cell should more than suffice to accommodate all the 13 000 vesicles as a single sub-membrane layer. With 10–20% of the vesicles concomitantly involved in the secretory process—say half of the vesicle surface in intimate contact with the chromaffin cell membrane the sodium influx and the consequent CA release should amount to less than one per mille of the CA content per secreting vesicle (1 per mille ~ 1000 CA molecules).

The figures calculated for the nerve impulse induced sodium ion flux across the contact area between the chromaffin cell membrane and the vesicle membrane are rather speculative—to say the least—based as they are on several uncertain assumptions. However, they seem to us to indicate the possibility of a nerve impulse induced sodium ion influx during the millisecond of depolarization that might suffice for a multivesicular fractional release of CA based on a cation exchange as discussed in previous papers (Uvnäs & Åberg 1980).

Next is the evaluation of the vesicles of the chromaffin cell as secondary instead of a primary event. A multivesicular fractional CA release on a cation exchange basis might seem difficult to reconcile with the many microscopical observations of exocytosis and expulsion of vesicular contents into the exterior of the chromaffin cell. One argument for an all or none response with a quantal release of whole vesicle packages might seem to be specially difficult to come around, namely that CA and other vesicle components are reported to appear in perfusates or plasma in the same mutual proportions as in the chromaffin cell.

However under the commonly prevailing experimental conditions it has not been possible to study the chronological order in which the different vesicle components are released. For the activation of the adrenal secretion humoral—usually acetylcholine or carbamylcholine—or nervous stimuli (1–10 Hz or more) of usually biologically high intensity have usually been employed. The consequence of a high stimulation intensity will be a rapid depletion of the CA vesicles. Let us for the sake of convenience assume that ~3000 vesicles are continuously engaged in the CA release. On nervous stimulation with a frequency of 20–30 Hz—a frequency commonly used—the vesicle stores will be depleted within 3 min. Already with these moderate stimulation frequencies a possible dissociation of CA release and a discharge of the remaining vesicle content will escape observation if—as usually done—sampling of perfusates and blood is made in 5–10–15-min periods or longer. With the high doses of acetylcholine and carbamylcholine and its secretory stimuli the possibilities to observe chronological detail of the release process have been eliminated. However in the few experiments where care has been taken to use stimuli of moderate intensity and (or) frequent sampling has been made, a temporal dissociation has been observed between CA release and the appearance of protein (Buckley et al. 1967; Karsner et al. 1967; Dixon et al. 1975). The retarded protein outflow has been interpreted as due to slow diffusion of proteins in the phenomenon might according to our interpretation as well be taken to indicate the subsequent emptying of CA depleted vesicles. Recently Edwards et al. (1979) reported that the output of β -hydroxytyramine was not proportional to the output of CA observed on splanchic nerve stimulation in conscious calves.

We therefore think that the available experimental data might be in keeping with the idea of a cation induced CA release as the primary event with the discharge of the remaining matrix of the vesicle as a second event. It should be pointed out that with the low impulse frequencies prevailing under physiological conditions (~1/s) and the considerable rate of resynthesis of CA assumed to exist (see Sjöberg et al. 1977) complete emptying of a vesicle CA store and its ultimate consequence—the expulsion of the vesicle matrix—might be a rather infrequent phenomenon (Fig. 1).

As pointed out by Sjöberg (1970) there are some

observations which do not fit the idea of a quantal secretion based on the total evacuation of CA vesicles. For example the quantal output of CA per nerve impulse from the cat adrenal gland depends both on stimulation frequency and on the total number of pulses i.e. the duration of stimulation. On stimulation of short duration there is an apparently linear increase on prolonged stimulation a decrease of the CA secretion. Such phenomena might be satisfactorily explained on the basis of a cation exchange dependent release of CA where the size of the released CA quanta will depend on the number of cations reaching the granule CA store and not on the total content of a vesicle.

Cation exchange: common principle for the release and release of biogenic amine We have previously shown that the release of histamine from mast cells can be explained as due to a cation exchange between extracellular sodium ions and intracellular histamine ionically bound to cationic sites (COO⁻ groups) in the granule matrix (for review see Ulfhåth 1977).

In the present paper we have described a cation (Na⁺) dependent release of CA from adrenal medullary granules in vitro and argued for a cation exchange dependent CA release being instrumental also on nerve impulse induced CA release in vivo.

We would in this connection like to call attention to the suggestion by Ulfhåth (1971) that also nervous transmitter release might be due to nerve impulse induced cation exchange. According to Folkow, Höglund & Lundberg (1967) experimental data indicate that only about 1% (400 out of 15000 molecules) of the NA content of one adrenergic granule is released per stimulus from the individual nerve varicosity provided that each varicosity releases transmitter every time an impulse arrives.

If the calculations of Folkow et al. are correct it follows that the smallest releasable quantum of NA does not correspond to the content of a transmitter vesicle but only to a small fraction thereof. Such fractional transmitter release from single or multiple vesicles might be explained on the basis of a cation exchange due to nerve impulse induced sodium flux across the nerve terminal-transmitter vesicle membranes at adrenergic as well as cholinergic nerve terminals. In fact preliminary in vitro observations suggest to us the occurrence of cation exchange dependent storage and release of nervous transmitters in nerve granules. These findings will be presented in forthcoming papers.

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Plasma concentration of neurotensin like immunoreactivity (NTLI) and lower esophageal sphincter (LES) pressure in man following infusion of (Gln¹)-neurotensin

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(Gln¹)-neurotensin was infused for 5 to 70 min at 3 different infusion rates (6, 12 and 18 pmol kg⁻¹ min⁻¹ respectively) in 19 male volunteers aged 26-47. The plasma concentration of neurotensin-like immunoreactivity (NTLI), the lower esophageal sphincter (LES) pressure, blood pressure, heart rate, ECG and blood glucose concentration were measured. The volunteers did not report any subjective effects during the infusion. Following infusion periods of 30 min or more the volunteers often reported bowel movements starting 5 min or more after cessation of the infusion. Neither blood pressure nor heart rate changed significantly. No changes were noted in the continuous ECG or in the blood glucose concentration. Apparent steady state levels of about 300 pM NTLI were reached at about 40 min during infusion of 12 pmol kg⁻¹ min⁻¹ (Gln¹)-neurotensin. In all volunteers the LES pressure was significantly reduced within 5 min of starting the infusion. In 6 volunteers 1 pmol kg⁻¹ min⁻¹ (Gln¹)-neurotensin was infused for 5 min. The LES pressure decreased significantly ($P < 0.01$) from 13.7 ± 1.3 mmHg to 5.3 ± 0.8 mmHg. The decrease in the LES pressure occurred at plasma NTLI concentrations of approximately 50 pM, at levels below those obtained in man after meal or the ingestion of fat. The present data further support the hypothesis that in man plasma neurotensin, or neurotensin metabolites, is an endocrine hormone involved in the postprandial regulation of the motor functions of the gastrointestinal tract.

Key words: Neurotensin, lower esophageal sphincter pressure.

In several species including man, immunocytochemical investigations have shown that neurotensin, tridecapeptide (Carraway & Leeman 1973, 1975), is stored in a specific type of cell (the N cell) which is present in the mucosa of the ileum and jejunum and, to a lesser extent, in the colon and duodenum (Carraway & Leeman 1976; Orri et al. 1976; Sandler et al. 1977; Helmstetter et al. 1977; Probst et al. 1977; Frijerko et al. 1977). Folkers et al. (1976) suggested that (Gln¹)-neurotensin may be the naturally-occurring peptide rather than neuroten-

sine. The biological tests which have been carried out so far show that the two peptides have the same biological activity both in vitro (Rökæus et al. 1977) and in vivo (Andersson et al. 1976, 1977). In anesthetized dogs higher concentrations of neurotensin-like immunoreactivity (NTLI) were found in blood draining from the ileum than in arterial blood (Marshford et al. 1978a) which indicates that neurotensin is released into the blood from the ileum. It has been shown in man that ingestion of a mixed meal caused a marked rise in the concentra-

tion of NTLI in blood (Besterman et al 1978, Mashford et al 1978b) and food appears to exert a major influence on the blood concentration of NTLI (Mashford et al 1978b). The ingestion of fat (Intralipid) is effective in increasing the plasma NTLI concentration whereas isocaloric amounts of amino acids (Vamin) and glucose have negligible effects (Rosell & Rokaeus 1979). Neurotensin administered by i.v. infusion in small doses to dogs inhibited gastric motility (Andersson et al 1977) and suppressed gastric acid secretion (Andersson et al 1976). It also increased blood flow in the gastrointestinal region and decreased it in subcutaneous adipose tissue (Rosell et al 1976). Taken together these data suggest that neurotensin or a metabolite thereof in the blood may be a hormone involved in the postprandial inhibition of gastric motility or gastric acid secretion and thus function as an enterogastrone (Mashford et al 1978a, Rosell & Rokaeus 1979). However, before neurotensin or a metabolite thereof can be ascribed such a physiological role it remains to be shown that NTLI concentrations in blood after the ingestion of fat are in the range found after the administration of doses of exogenous neurotensin capable of inhibiting motility in the gastrointestinal tract or gastric acid secretion in humans.

The purpose of the present investigation was to see if iv infusion of (Gln)-neurotensin has any effects on the lower esophageal sphincter (LES) pressure in man at NTL plasma concentrations which have been reported after ingestion of fat. The LES pressure was considered to be a suitable parameter to measure since ingestion of a fatty meal is known to cause a pronounced decrease in the LES pressure in man (Nebel & Castell 197). The effects of administration of neurotensin or (Gln)-neurotensin to man have not been reported earlier. Therefore on the basis of data from toxicity tests in animals it was decided to monitor cardiovascular function by electrocardiography, blood pressure measurements and heart rate determination. Furthermore the plasma glucose concentration was measured.

MATERIAL AND METHODS

The studies were performed in 19 male volunteers aged 26-47 with no symptoms of gastrointestinal disease or history of abdominal surgery. The volunteers were asked to avoid eating fat in the evening before the experimental

day and the experiment were started after a fasting period of at least 6 h between 9 a.m. and p.m. After a resting period of at least 30 min an indwelling cannula was inserted into the antecubital vein of the arm for blood sampling and another cannula was inserted into the other arm for the i.v. infusion of (Gln)-neurotensin.

Systolic and diastolic blood pressures were measured by the cuff method at 5 min intervals from 10 min before to 40 min after the i.v. infusion. A continuous ECG, approximately lead CR₄, was registered by an ink jet recorder paper speed 10 mm/s throughout the investigation. Blood samples were taken at 5 min intervals from 3 min before to 40 min after the i.v. infusion of (Gln)⁷-neurotensin. The samples were immediately placed on ice and later centrifuged at 4°C. Two portions of each plasma sample were frozen and stored at -20°C until analysed. (Gln)⁷-neurotensin was obtained from the same batch (No. 14609, Institute for Biomedical Research, University of Texas at Austin, USA) as has been used for previous tests (T. Malmfors, unpublished). The substance was dissolved in sterile water under aseptic conditions and the solution was filtered (Millipore, pore size 0.2 µm) directly into 5 ml vial (10 µg/ml) at 4°C (Karlensjö & Nilsson 1973). The biological activity of a sample of this solution was compared with the activity of (Gln)⁷-neurotensin in samples from the same batch which had not been filtered. The biological activity was tested on the rat fundus strip (Rökæus *et al.* 1977) and was found to be the same for the two samples. Similarly, the vasopressor gave the same values in the radioimmunoassay for neurotensin. Plasma glucose was measured in duplicate (Glox, Abbot). Immunoreactive neurotensin (NTLI) was determined in duplicate in each plasma sample within 8 weeks after the experiment.

The method used in the radioimmunoassay of neurotensin will be presented in detail elsewhere (to be published) and therefore only a brief description of the method is given here. The antiserum (0-7781) was raised in rabbits by injection of neurotensin coupled to bovine serum albumin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Goudfriend Levine & Fawcett 1964). The antigen was injected together with Freund's complete adjuvant at 3-8 week intervals. Three antiserum reacts with neurotensins n (NT), NT (1-11) (Gln) NT and (Gln) NT (1-11) but not with NT (4-13) or smaller C terminal fragments of NT. It shows no cross-reactivity with the gastrin inhibitory polypeptide (GIP), the pancreatic secretory peptide (VIP), secretin, cholecystokinin-33, cholecystokinin-39, pancreatic glucagon, substance P, bombesin, somatostatin, the porcine pancreatic polypeptide, amylin, bombesin, galanin-17, gastrin-34 or with tryptophan pentadecapeptide. Synthetic neurotensin (Beckman) labelled with ^{125}I using the chloramine T method (Hunt & Greenwood 1962). The assay can measure levels down to 4 pM. Experimental data indicate that some of the actions ascribed to neurotensin (e.g. constriction of adipose tissue) may be due to neurotensin metabolites (Rossell et al 1978). Since the radio-immunoassay is specific enough to differentiate between neurotensin and its metabolic pharmacokinetic treatment of the plasma concentration data is limited to evaluation of steady state concentrations and the terminal elimination

Table 1 Plasma glucose concentration during infusion of (Gln)-neurotensin

| exp no | Inf of (Gln)-NT | | | Plasma glucose (mM) | | | | |
|----------------|-----------------|-----|-----|---------------------|--------|--------|--------|--------|
| | pool | kg | min | Control | 10 min | 20 min | 30 min | 70 min |
| 1 | 6 | | | 8.5 | 9.1 | 8.1 | 8.4 | 8.8 |
| 2 | 6 | | | 7.4 | 7.1 | 7.6 | 6.9 | 6.4 |
| 3 | 12 | | | 4.8 | 4.6 | 4.3 | 5.0 | 5.1 |
| 12 | 1 | | | 3.9 | 3.8 | 3.7 | 3.3 | 3.6 |
| 77 | 1 | | | 4.5 | 3.7 | 4.1 | 4.0 | 3.9 |
| 71 | 6 | | | 4.4 | 4.8 | 4.9 | | |
| 72 | 6 | | | 9 | 8 | 7.8 | | |
| 712 | 6 | | | 3.4 | 3.3 | 3.5 | | |
| low for 6 pool | kg | min | | | | | | |
| 7-74 | | | | 9.3 | 9.4 | 9.4 | | |

steps (i) Calculation of $\frac{1}{2}$ as made from the measured concentrations after deduction of the background concentration. Thus the $\frac{1}{2}$ reflects the pharmacological elimination of the administered neurotensin. The half-life was calculated from the plasma NTLI concentrations versus time curve by the method of least squares. The mean plasma NTLI level in 3 samples from the control period preceding the infusion period was taken as the background.

To measure the lower esophageal sphincter (LES) pressure esophageal manometry was performed in one series of experiments. A 3-lumen manometric catheter assembly consisting of polyethylene catheters (inner diameter 1.5 mm, outer diameter 2.5 mm) with lateral openings 5 cm apart, connected to external pressure transducers (Elema, Schönder JNJ 14). Pressure changes were observed on an oscilloscope and recorded on a multichannel photographic recorder (Elema Schönder Oscillograph 3006/DL). Each catheter was perfused with distilled water at constant rate of 1.2 ml/min by Harvard constant infusion pump. After the subject in the supine position, the catheter assembly was passed by the transnasal route into the stomach and then slowly withdrawn until the middle aperture was recording the LES pressure. Throughout each study the position of this aperture was maintained at the point of maximum pressure. LES pressure was recorded continuously of mercury using a zero resting intragastric pressure as zero reference. The average pressure was determined from the recording during each minute of the test. In another series of experiments polyethylene catheter (inner diameter 3.5 mm) with 4 side holes (1.2 mm) at the same level was perfused at constant rate of 3 ml/min. It was passed by the transnasal route and withdrawn during suspended respiration at constant rate of 1 cm/min from the fundus of the stomach to the esophagus. The intragastric pressure was used as zero reference. The data as connected to the external transducer and pressure changes were observed on an oscilloscope and recorded on the multichannel photographic recorder. Each pull-through was carried out at 2.5 intervals.

Statistical evaluation of the data was performed with the Wilcoxon signed rank test and Student's *t*-test. The results are reported as mean \pm standard error of the mean (M \pm S.E.).

RESULTS

Cardiovascular action plasma glucose and subject effect

(Gln)-neurotensin was administered i.v. for periods of 5 to 70 min at 3 different infusion rates: 6, 1 and 18 pmol \times kg \times min⁻¹ respectively. The systolic and diastolic blood pressures did not change significantly during or after the infusion periods. Thus, the mean decrease in systolic and diastolic blood pressures 5 min after the start of the infusion of 12 pmol \times kg \times min⁻¹ was 5.9 and 3.2 mmHg respectively (mean \pm S.E.). The mean change in heart rate was 0.7 beats \times min⁻¹. Arrhythmias were not observed and no ST or T changes were noted in the recorded lead CR₂. The plasma glucose concentration did not change significantly (Table 1). The volunteers did not report any subjective effects during the infusion period. However following infusion periods of 30 min or more the volunteers often reported bowel movements starting 5 min or longer after cessation of the infusion and two of the volunteers had an urgent need to defecate within 30 min.

Plasma level of NTLI

The plasma NTLI concentration curves following infusion of 12 pmol \times kg \times min⁻¹ (Gln)-neurotensin in two subjects are shown in Fig. 1. The initial part of the curves resemble zero order infusion curves. Apparent steady state levels of about 300 pM were reached at about 40 min. The elimination slopes following the cessation of the infusions appeared to be multiexponential. This was the case following infusion periods of 70 min ($n=3$). However following shorter infusion periods the elimina-

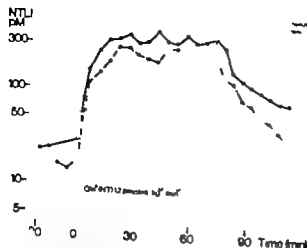


Fig. 1 Plasma concentration of neurotensin-like immunoreactivity (NTLI) in two subjects in response to intravenous infusion of (Gln)-neurotensin $1 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$. Vertical lines indicate the infusion period.

tion slopes seemed to be monoexponential. In experiments with infusion periods 5–30 min the $t_{1/2}$ calculated on the first 4 to 6 post infusion determinations of plasma NTLI concentration was $7.3 \pm 0.6 \text{ min}$ ($n=7$). In 5 experiments the infusion period was 70 min. In those experiments the $t_{1/2}$ was calculated on the first 4 to 8 NTLI determinations to be $9.4 \pm 0.9 \text{ min}$ ($n=5$). The $t_{1/2}$ calculated on the last 3–6 determinations was $19.9 \pm 1 \text{ min}$ ($n=3$).

Lower esophageal sphincter pressure

13 pressure measurements in the lower esophageal sphincter were carried out in 17 volunteers receiving $6\text{--}18 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$ (Gln)-neurotensin i.v. during 5–70 min. In all volunteers the LES pressure was reduced significantly within the first 5 min of the infusion period. Thereafter despite continued infusion of (Gln)-neurotensin there was a gradual but only partial return of the LES pressure to preinfusion values (Fig. 2).

In 6 volunteers $12 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$ (Gln)-neurotensin was infused i.v. for 5 min. The LES pressure decreased significantly ($P < 0.01$) from $13.7 \pm 1.3 \text{ mmHg}$ to $5.3 \pm 0.8 \text{ mmHg}$ with a rapid return to preinfusion values during the post infusion period (Fig. 3). Thus in comparison with the preinfusion values there was no statistically significant reduction of the LES pressure 4 min after the cessation of the infusion. The decrease in LES pressure occurred at plasma NTLI concentrations of approximately 50 pM (Fig. 3). Apart from reducing the LES pressure (Gln)-neurotensin infusion also stabilized the nonrespiratory movements of the

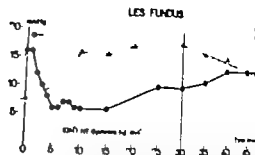


Fig. 2 LES pressure (Δ) and plasma concentration of NTLI (\circ) in one subject in response to intravenous infusion of (Gln)-neurotensin $12 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$ for 30 min. Vertical lines indicate the infusion period.

resting LES pressure seen in some volunteers. In 4 volunteers $1 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$ (Gln)-neurotensin was infused i.v. for 10 min. The pressure measured with the continuous recording technique was reduced from $31.5 \pm 4 \text{ mmHg}$ to $19.4 \pm 4 \text{ mmHg}$ ($n=4$) (Fig. 3).

DISCUSSION

The present series of experiments on healthy volunteers show that i.v. administration of (Gln)-neurotensin at low rates ($6\text{--}18 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$) produces pronounced effect in the gastrointestinal tract. The LES pressure decreased profoundly and the subjects frequently reported bowel movements.

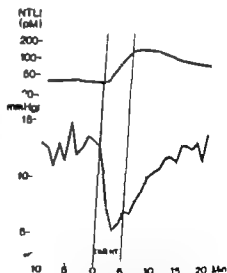


Fig. 3 Plasma concentration of NTLI (upper curve) and lower esophageal sphincter (LES) pressure in response to intravenous infusion of $1 \text{ pmol} \times \text{kg} \times \text{min}^{-1}$ for 5 min. The shaded area indicates the standard error of the mean ($n=6$). Vertical lines indicate the infusion period.

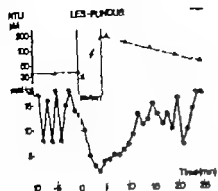


Fig. 4 Plasma concentration of NTLI and the LES pressure in one subject. Thin vertical lines indicate intravenous infusion of (Gln)-neurotensin 12 pmol.

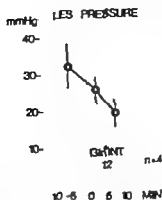


Fig. 5 The LES pressure as recorded with the continuous pull-through technique. Vertical lines indicate intravenous infusion of (Gln)-neurotensin 12 pmol. Mean \pm S.E. are indicated ($n=4$).

after cessation of the infusion and even an urgent need to defaecate. No significant changes in blood pressure, heart rate or in the continuously registered ECG are noted in this dose range. Moreover, the plasma glucose concentration did not change. The actions in humans resemble the actions reported in dogs following i.v. infusions of neurotensin or (Gln)-neurotensin in the same dose range (Andersson et al. 1976, 1977). Thus, studies carried out so far show that in both man and dog the most sensitive target organ for neurotensin is the gastrointestinal tract (Mashford et al. 1978a). Infusion of (Gln)-neurotensin increased the plasma NTLI concentration in an approximately dose-dependent manner. Infusion of 12 pmol \times kg \times min resulted in a plasma NTLI concentration of about 300 pM and after 18 pmol \times kg \times min in one subject a level of about 600 pM was reached. These concentrations of NTLI are in the same order of magnitude as has been measured in man after a meal. Using the same technique as has been used in the assays in the present series, Mashford et al. (1978b) found that ingestion of a meal after fasting for 24 h resulted in a mean plasma NTLI concentration of \sim 33 pM with a maximal value of about 600 pM in one subject. Consequently, the effects on the LES pressure observed in the present investigation following infusion of 6–18 pmol \times kg \times min occur at plasma NTLI concentrations which are found in man under physiological conditions.

Clearly (Gln)-neurotensin does not have pronounced cardiovascular actions in man. On the basis of experiments on rat (Carraro & Leeman (1973))

suggested that neurotensin is a potent hypotensive and hyperglycaemic peptide. However, to induce hypotension in dogs, neurotensin has to be infused i.v. at about 10 times the dose necessary to induce inhibition of the motor activity in the gastro-intestinal tract (Rosell et al. 1976; Andersson et al. 1977). Similarly, to increase the plasma glucose concentrations in dogs, neurotensin and (Gln)-neurotensin must be administered in approximately the same doses that cause hypotension (Rosell et al. 1976). In the present experiments, blood pressure, heart rate and plasma glucose level did not change. These quantitative comparisons indicate that in man, as in the dog, there is a clear-cut difference between the doses that produce gastrointestinal effect on the one hand and general cardiovascular effects and increased plasma glucose concentrations on the other.

The elimination of NTLI might possibly be multiphasic. However, a pharmacokinetic analysis of the plasma NTLI data should be postponed until it is known whether or not the radioimmunoassay is specific for administered peptide or also measures some of its metabolite(s) which may have different elimination rates.

The lower esophageal sphincter pressure was measured with the perfused catheter assembly positioned with lateral openings in the esophagus, the LES and the fundus of the stomach respectively or with pull-through technique with one perfused catheter. Both techniques have their advantages and disadvantages. The pull-through technique will measure LES pressure only momen-

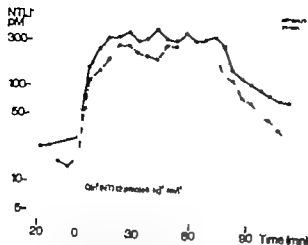


Fig. 1 Plasma concentrations of neurotensin-like immunoreactivity (NTLI) in two subjects in response to intravenous infusion of (Gln)-neurotensin $1 \text{ pmol} \times \text{kg} \times \text{min}$. Vertical lines indicate the infusion period.

tion slopes seemed to be monoexponential. In experiments with infusion periods 5–30 min the $t_{1/2}$ calculated on the first 4 to 6 post infusion determinations of plasma NTLI concentrations was $7.3 \pm 0.6 \text{ min}$ ($n=7$). In 5 expts the infusion period was 70 min. In those expts $t_{1/2}$ was calculated on the first 4 to 8 NTLI determinations to be $9.4 \pm 0.9 \text{ min}$ ($n=5$); the $t_{1/2}$ calculated on the last 3–6 determinations was $19.9 \pm 1 \text{ min}$ ($n=3$).

Lower esophageal sphincter pressure

13 pressure measurements in the lower esophageal sphincter were carried out in 12 volunteers receiving $6\text{--}18 \text{ pmol} \times \text{kg} \times \text{min}$ (Gln)-neurotensin i.v. during 5–70 min. In all volunteers the LES pressure was reduced significantly within the first 5 min of the infusion period. Thereafter despite continued infusion of (Gln)-neurotensin there was a gradual but only partial return of the LES pressure to preinfusion values (Fig. 2).

In 8 volunteers $17 \text{ pmol} \times \text{kg} \times \text{min}$ (Gln)-neurotensin was infused i.v. for 5 min. The LES pressure decreased significantly ($P < 0.01$) from $13.7 \pm 1.3 \text{ mmHg}$ to $5.3 \pm 0.8 \text{ mmHg}$ with a rapid return to preinfusion values during the post infusion period (Fig. 3). Thus in comparison with the preinfusion values there was no statistically significant reduction of the LES pressure 4 min after the cessation of the infusion. The decrease in LES pressure occurred at plasma NTLI concentrations of approximately 50 pM (Fig. 3). Apart from reducing the LES pressure (Gln)-neurotensin infusion also stabilized the nonrespiratory movements of the

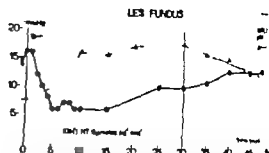


Fig. 2 Plasma concentration of NTLI (Δ) and LES-pressure (\bullet) in one subject in response to intravenous infusion of (Gln)-neurotensin $6 \text{ pmol} \times \text{kg} \times \text{min}$ for 10 min. Vertical lines indicate the infusion period.

resting LES pressure seen in some volunteers (Fig. 4). In 4 volunteers $12 \text{ pmol} \times \text{kg} \times \text{min}$ (Gln)-neurotensin was infused i.v. for 10 min. The LES pressure measured with the continuous perfusion technique was reduced from $31 \pm 2 \text{ mmHg}$ to $19 \pm 4 \text{ mmHg}$ ($n=4$) (Fig. 5).

DISCUSSION

The present series of experiments on healthy volunteers show that i.v. administration of (Gln)-neurotensin at low rates ($6\text{--}18 \text{ pmol} \times \text{kg} \times \text{min}$) produces pronounced effects in the gastrointestinal tract. The LES pressure decreased profoundly and the subjects frequently reported bowel movements.

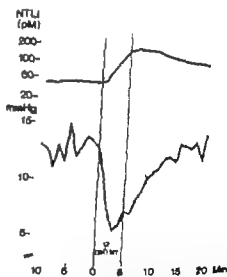


Fig. 3 Plasma concentration of NTLI (upper curve) and lower esophageal sphincter (LES) pressure (lower curve) in response to intravenous infusion of $1 \text{ pmol} \times \text{kg} \times \text{min}$ for 5 min. The shaded area indicates the standard error of the mean ($n=6$). Vertical lines indicate the infusion period.

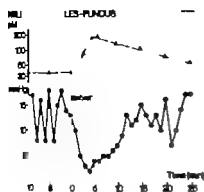


Fig. 4 Plasma concentration of NTLI and the LES pressure in one subject. This vertical lines indicate intravenous infusion of (Gln)-neurotensin 12 pmol/kg.

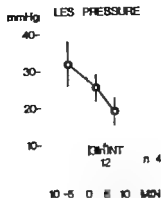


Fig. 5 The LES pressure as recorded with the continuous pull-through technique. Vertical lines indicate intravenous infusion of (Gln)-neurotensin, 12 pmol/kg \times min. Means \pm S.E. are indicated ($n=4$).

After cessation of the infusion and even an urgent need to defecate. No significant changes in blood pressure, heart rate or in the continuously registered ECG were noted in this dose range. Moreover the plasma glucose concentration did not change. The actions in humans resemble the actions reported in dogs following i.v. infusions of neurotensin or (Gln)-neurotensin in the same dose range (Andersson et al. 1976, 1977). These studies carried out so far show that in both man and dog the most sensitive target organ for neurotensin is the gastrointestinal tract (Mashford et al. 1978b). Infusion of (Gln)-neurotensin increased the plasma NTLI concentrations in an approximately dose-dependent manner. Infusion of 1 pmol \times kg $^{-1}$ \times min resulted in a plasma NTLI concentration of about 300 pM and after 18 pmol/kg \times min in one subject a level of about 500 pM was reached. These concentrations of NTLI are in the same order of magnitude as has been measured in man after meal using the same method as has been used in the assays in the present series. Mashford et al. (1978b) found that ingestion of meal after fasting for 24 h resulted in a mean plasma NTLI concentration of 233 pM, with a maximal value of about 600 pM in one subject. Consequently the effects on the LES pressure observed in the present investigation following infusion of 6–18 pmol/kg \times min occur at plasma NTLI concentrations which are found in man under physiological conditions.

Evidently (Gln)-neurotensin does not have pronounced cardiovascular actions in man. On the basis of experiments on rats, Carraway & Leeman (1973)

suggested that neurotensin is a potent hypotensive and hyperglycemic peptide. However to induce hypotension in dogs neurotensin has to be infused i.v. at about 10 times the dose necessary to induce inhibition of the motor activity in the gastro-intestinal tract (Rosell et al. 1976; Andersson et al. 1977). Similarly to increase the plasma glucose concentrations in dogs neurotensin and (Gln)-neurotensin must be administered in approximately the same doses that cause hypotension (Rosell et al. 1976). In the present pts. blood pressure, heart rate and plasma glucose level did not change. These quantitative comparisons indicate that in man, as in the dog, there is a clear-cut difference between the doses that produce gastrointestinal effects on the one hand and general cardiovascular effect and increased plasma glucose concentration on the other.

The elimination of NTLI might possibly be multiphasic. However a pharmacokinetic analysis of the plasma NTLI data should be postponed until it is known whether or not the radioimmunoassay is specific for administered peptide or also measures some of its metabolite(s) which may have different elimination rates.

The lower esophageal sphincter pressure was measured with the perfused catheter assembly positioned with lateral openings in the esophagus, the LES and the fundus of the stomach respectively or with pull-through technique with one perfused catheter. Both techniques have their advantages and disadvantages. The pull-through technique will measure LES pressure only momen-

tarly. The resting LES pressure fluctuates (Bloom et al 1974) and is therefore difficult to determine with the pull through technique. On the other hand with continuous registration of LES pressure the pressures are lower due to the low perfusion rate and the technique is not suitable for dose response studies but merely to find out if an effect can be registered or not. However in the present study a decrease of the LES pressure after infusion of (Gln)-neurotensin could be demonstrated with both techniques.

Nebel & Castell (1977) showed that ingestion of a fatty meal caused a pronounced decrease in the LES pressure in man. This is interesting in view of our finding that fat is an important stimulus for the release of NTI I (Rosell & Rönkäus 1979). Therefore neurotensin or a neurotensin metabolite might be responsible for the decrease in the LES pressure after a fatty meal. Several hormones released after food intake such as gastrin, secretin, cholecystokinin and glucagon have been shown to influence the LES pressure (Nebel & Castell 1977, Giles et al 1969, Castell & Harris 1970, Cohen & Lipshutz 1971, Resin et al 1973, Jaffar et al 1974) but it has not been established whether these hormones are of any physiological importance in the regulation of the LES pressure (Grossman 1973, Cohen & Lipshutz 1974, Sturdevant 1974, Henderson et al 1978).

We thank Professor John Warren and his staff for placing laboratory facilities at our disposal.

The study has been supported by the Swedish Medical Research Council (grant No. 3518), AB Astra Research Foundation, Stiftelsen Ruth och Richard Julins fund and the Robert A. Welch Foundation and approved by the Ethical Committee at Karolinska Institute.

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Firing behaviour in nerve cell models with a two-state pore system

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SKAUGEN E.: Firing behaviour in nerve cell models with a two-state pore system. *Acta Physiol Scand* 1980; 109: 377-392. Received 16 Nov 1979. ISSN 0001-6777. Institute of Physiology and Informatics, University of Oslo, Norway.

The firing behaviour of simple nerve cell models with a two-state pore system was investigated by computer simulation. The pores were assumed to open and close randomly with the probabilities for opening and closing calculated from the equations found by Hodgkin and Huxley for the squid giant axon. The cell models had a cell body and an axon with an initial segment with a smaller diameter. Both the case of a uniform membrane with constant pore densities all over the cell and the case of a cell body membrane with only leakage conductance (passive membrane) were investigated. The results indicate that the firing behaviour of small nerve cells may be significantly influenced by the finite number of pores in the initial segment. In contrast to the original Hodgkin-Huxley equations which give a very non-linear frequency-current relationship in such nerve cell models, a fairly linear relationship over a large current range was found in many cases. It was estimated that the diameter of the initial segment must be less than approximately 1 μm and the length larger than half a space constant, in order to obtain a current frequency relationship significantly different from that predicted by the original Hodgkin-Huxley equations.

Key words. Hodgkin-Huxley membrane, nerve membrane model, stochastic pores

In two earlier papers it was shown that a finite number of pores or ionic channels in the nerve membrane could give stochastic fluctuations of the membrane conductances and the membrane potential (Skaugen & Walløe 1979; Skaugen 1980). Only the case of a space-clamped, uniform nerve membrane was considered. For sufficiently small pore numbers, less than approximately 20 000 pores, the firing behaviour of this space-clamped membrane was significantly influenced by these fluctuations. Here I try to indicate how the stochastic fluctuations due to a finite number of pores may influence the firing behaviour of physiologically operating nerve cells. This was studied by simulating on computer simple nerve cell models with a certain density of pores. The total number of pores in a nerve cell is much larger than the number 20 000 indicated above, but what is important here is the number of pores around the site for spike generation. Usually this site is connected to the axon, and has a small diameter compared to the rest of the

cell. This will partly insulate it from the rest of the cell that is the axial resistance between the site for spike generation and the cell body or the dendrites, and between this site and the axon is not insignificant compared to the membrane resistance of this site in the resting state. The pore density in the initial segment is not known and its value can only be guessed at. In different nerve fibres pore densities from 2.5 pores/ μm^2 to several hundred pores/ μm^2 have been measured (Colquhoun et al. 1972; Keynes & Rojas 1974).

I have chosen to simulate a simple model of nerve cells in order to study the "pure" effects of a finite number of pores without getting these effects mixed up with for instance the effects of a complicated dendritic network (with time delays reflecting of action potentials etc. (Fernández 1971)).

A realistic model of a nerve cell demands a detailed knowledge of the membrane properties of the whole neuron. The membrane properties at the site for spike generation and at areas adjacent to it

| subset No. 1, 2, 3 | Length of initial segment divided by space constant $\lambda/2$ | Used in figures |
|--------------------------|--|------------------|
| | 0.18 | —, 3, 5, 6, 1 |
| | 0.41 | 2 to 10 and 1 |
| 1 | 0.91 | to 6, 9, 11, 1 |
| | 0.18 | —, 3, 5, 6, 12 |
| | 0.41 | to 10 and 1 |
| 2 | 0.91 | 2 to 6, 9, 11, 1 |
| 1 | 0.77 | 4 |
| | 0.41 | 8, 9 |
| 2 | 0.91 | 9 |
| | 0.77 | 10 |
| | 0.18 | 10 |
| 1 | 0.64 | 11 |

a fraction of the injected current (in the cell body), for a few combinations of the size and the area of the cell, and of the pore densities in the initial segment. I either assumed that the density of pores in the cell body was equal to that in the initial segment, or that the nerve membrane of the cell body was purely passive. In the latter case a specific leakage conductance double of that in the initial segment was assumed in order to compensate for the lack of the potassium current, while the equilibrium potential for the leakage current was assumed equal to the resting potential. It seems that the densities of nerve cells do not in general conduct sensory impulses, which may mean that the nerve membrane behaves more and more like a passive membrane as one goes from the initial segment towards the dendritic branches (which can be explained by decrease in the pore densities).

The two cases considered here are thus extreme cases and the behaviour of a real nerve cell would be expected to lie between the behaviour shown by these two examples.

THEORY

The basis for these calculations is the now widely accepted assumption that the nerve membrane conducts sodium and potassium ions through pores, or ionic channels, and that the opening and closing of these pores are governed by stochastic process. As in the earlier papers no other types of electrical noise than conductance fluctuations are considered in the calculations (Slaugen & Walløe 1979; Slaugen 1980). The detail of the assumptions used here for the pore mechanisms were given in the first of these papers. For the readers' convenience these assumptions are restated here.

1 The nerve membrane conducts ions through pores. In addition there is leakage conductance which is constant.

2 A particular pore conducts only certain type of ion, either sodium or potassium ions.

3 All pores which conduct the same type of ion have the same conductance when in the open state.

4 There is no coupling between neighboring gates or pores.

5 Each pore has four gates. The pore is open only if all four gates are open.

6 The transition probabilities of gates from one state to another—either from shut to open, or from open to shut—are only potential dependent and given by the α and β parameters of the H-H equations.

7 The transition times of the gates are so short that they can be neglected. (The transition time is the time used by the gate to go from the shut to the open position or vice versa.)

The rest of the assumptions and the equations necessary to completely specify the space-clamped version of the model are given by Hodgkin and Huxley paper (Hodgkin & Huxley 1952).

In the modelling of whole nerve cell the usual method of dividing the cell into a number of compartments is used. I assume here that the compartments are cylindrical, the j -th compartment having diameter d_j and length l_j . Most often five compartments were used ($j=1-5$). Fig. 1 shows an approximation to typical crustacean nerve cell with cell body tapering off to an axon with an initial segment which has smaller diameter than the rest of the axon.

One of the compartments, usually the middle one represented the main part of the initial segment. This compartment is termed the stochastic compartment, or the s -th compartment. In order to simplify the model sufficiently only this compartment was assumed to have finite number of pores. The initial segment has the smallest diameter and the smallest number of pores of the compartments. The inclusion of the effects of finite pore numbers for all compartments would increase enormously the number of pores taken into account, and it would make the computer time needed prohibitively because the

Table 1 *Different nerve cell models used in the calculations*

| Model | Dimensions of compartments when $R_i = 110 \Omega\text{-cm diameter/length } (\mu\text{m})$ | | | | | Value of δ for N $N_{\infty} = N_{\infty} =$ | | Pore density for $N_{\infty} = N_{\infty}$ | |
|-------|--|------|------|------|----------------------|--|-------|---|------|
| | Compartment no | | | | | 1 000 | 100 | 1 000 (pores/ μm^2) | 100 |
| | 1 | 3 | 4 | 5 | $\eta = \eta/\delta$ | | | | |
| A | 16 | 0.43 | 0.2 | 0.33 | 0.43 | 1.0 | 1.0 | 400 | 50 |
| | 2.92 | 0.39 | 9 | 7.97 | 5.85 | | | | |
| B | 1.77 | 0.25 | 0.13 | 0.19 | 0.25 | 1.0 | 1.0 | 400 | 50 |
| | 5 | 1 | 5 | 5 | III | | | | |
| C | 0.74 | 0.15 | 0.07 | 0.11 | 0.15 | 1.0 | 1.0 | 400 | 50 |
| | 8.5 | 1.7 | 8.5 | 8.5 | 17.0 | | | | |
| A | 6.84 | 1.37 | 0.68 | 1.03 | 1.37 | 1.0 | 0.01 | 0.003 | 15.9 |
| | 79.2 | 5.85 | 79 | 79 | 58.5 | | | | |
| B | 4.00 | 0.80 | 0.40 | 0.60 | 0.80 | 1.0 | 0.03 | 0.003 | 15.9 |
| | 50 | 10 | 50 | 50 | 100 | | | | |
| C | 34 | 0.47 | 0.3 | 0.35 | 0.47 | 1.0 | 0.01 | 0.003 | 15.9 |
| | 85 | 17 | 85 | 85 | 170 | | | | |
| D | 3.17 | 0.63 | 0.3 | 0.48 | 0.63 | 1.0 | 0.03 | 0.0032 | 15.9 |
| | 63 | 13 | 63 | 63 | 176 | | | | |
| E | 1.4 | 0.80 | 0.40 | 0.60 | 0.80 | 1.0 | 0.012 | 0.003 | 15.9 |
| | 50 | 10 | 50 | 50 | 100 | | | | |
| F | 7.5 | 0.47 | 0.3 | 0.35 | 0.47 | 1.0 | 0.03 | 0.003 | 15.9 |
| | 85 | 17 | 85 | 85 | 170 | | | | |
| G | 4.00 | 0.80 | 0.40 | 0.60 | 0.80 | 0.5 | 0.064 | 0.0064 | 15.9 |
| | 50 | 10 | 50 | 50 | 100 | | | | |
| H | 4.00 | 0.80 | 0.40 | 0.60 | 0.80 | 0.2 | 0.16 | 0.016 | 15.9 |
| | 50 | 10 | 50 | 50 | 100 | | | | |
| I | 34 | 0.47 | 0.3 | 0.35 | 0.47 | 0.5 | 0.064 | 0.0064 | 15.9 |
| | 85 | 17 | 85 | 85 | 170 | | | | |

All models which can be transformed into each other by the equivalent transformations given by Eq. (4) are given the identification letter. The pore density given in the table is the density in the initial segment (compartment no. 1). The k of this compartment in units of the space constant λ is also shown: $k \sim (d/(8g_i R_i))^{1/2}$.

are obviously of special interest. Unfortunately the site for spike generation is generally of small dimensions compared to the rest of the cell and it is virtually impossible to measure the membrane properties here with the present-day techniques.

For a few large nerve cells the membrane properties of either the axon or the cell body have been investigated by the voltage clamp method. Well-known examples are the squid giant axon (Hodgkin & Huxley 1952), the myelinated nerve fibre of toad (Frankenhaeuser & Huxley 1964) and the myelinated nerve fibre of frog (Dodge 1963).

I have here chosen the Hodgkin-Huxley equations for the giant axon in squid as a basis for the calculations, as in the preceding papers (Skaugen & Walloe 1979; Skaugen 1980). This choice is rather arbitrary; there are a few other sufficiently detailed sets of equations for nerve membranes to choose from (Frankenhaeuser & Huxley 1964; Dodge 1963; Connor & Stevens 1971). The choice done here may

be defended on the grounds that I am most interested in the region of a nerve cell where the action potential is generated. These regions are unmyelinated and only the H-H equations describe an unmyelinated nerve fibre. Moreover the H-H equations are the simplest set of equations of the different sets we can choose from. In practice this means a considerably shorter computational time than could be obtained by using any of the other sets of equations.

Even using the H-H equations the present models were so complicated and time-consuming, mainly because of the introduction of a finite pore number, that only a limited number of possible combinations of parameters could be explored. A simple nerve cell with a cell body tapering off to an axon with an initial segment of smaller diameter than the rest of the axon was taken as the basic form of the nerve model. The data are in most cases presented as the mean firing frequency (in the axon).

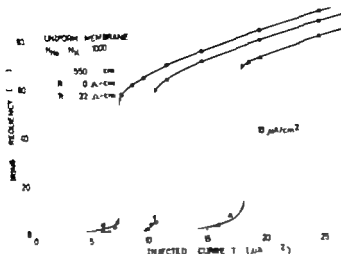


Fig. 3. Mean firing frequency as function of injected current for different values of R_i for model B in the case of uniform membrane and $N_m = N_h = 1000$. The curve for $R_i = 22$ ohm-cm differs for $10 \mu A/cm^2$ less than true value.

where the symbols S and N indicate that E_n depends on the total number N of pores in the n-th compartment and upon a stochastic process S. The only difference from the previous models is that here the absolute values of the capacitance, the current, and the conductance are used, and that the "injected" current depends upon the potential in neighbouring compartments.

The values of all parameters used in these equations are constant and equal to their values in the H-H equations, except the rate constants and the maximal, specific membrane conductances. The values of all the rate constants are multiplied by ϕ and the values of the maximal conductances are multiplied by η . This transformation has been suggested by Huxley (1959) and it was used in former paper (Skaggs 1980).

In the model the compartments are connected through a parameter l_j (or l_j) in Eq. (1). It is set equal to the current entering the j-th compartment. This current is the sum of the currents from the neighbouring compartments and the actual injected current. The latter is set equal to zero, except for the first compartment, the cell body. When I later speak of the injected current, we always mean the specific current injected in the cell body. It is the total current injected here divided by the membrane area of the first compartment. The current l_j in Eq. (1) is then given by

$$l_j = (E_j - E_n) g_{jn} + (E_j - E_{j+1}) g_{j,j+1} + I_j \quad (2)$$

where

$$I_j = \frac{1}{A_j} \left(\frac{1}{\phi} \frac{dE_j}{dt} + \frac{1}{\eta} \frac{dQ_j}{dt} \right) \quad j = 1, 2, \dots, J$$

$$E_n = E_{n-1} - E_d$$

$$I_{j-1} = I_j d_{j-1} d$$

where A_j is the membrane area of the first compartment, and R_i is the specific internal resistance. The parameter g_{jn} is the conductance between compartments j and n . The first compartment, the cell body, can be connected to the resting potential E_n by conductance g_{n0} in order to simulate the loading of the cell body by an extensive system of dendrites. But in the cases presented here g_{n0} was set equal to zero and the soma and the dendrites were lumped together as one long compartment. In similar way the last compartment, which is part of the axon, is loaded by conductance g_{j0} in order to simulate the continuation of the axon.

This model was tested against models where short dendritic tree and longer axon were included, but the results were not significantly different. The use of only one stochastic compartment probably introduced larger error. I did not, however, test the model against model with very extensive dendritic tree where appreciable time delay effect might be expected as may for instance be the case in many motor neurons. I should perhaps emphasize here that we are only interested in the firing behaviour of the nerve cell, mostly the firing frequency as function of the injected current and not, for instance, the conduction velocity of the action potential down the axon.

For given geometrical configuration, with the diameters d_j and the lengths l_j of the cylindrical compartments specified, the pore numbers N_m and N_h are given if the pore densities ρ_m and ρ_h are known, $N_m = \pi d_j l_j \rho_m$ and $N_h = \pi d_j l_j \rho_h$ where j indicates the stochastic compartment in the model we need not specify the pore densities, but the pore numbers must be known. With these numbers the geometry of the cell and all parameters in the H-H equations given the model is completely specified.

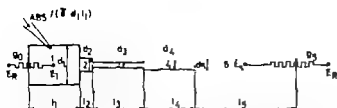


Fig. 1. Diagram of nerve cell model with 5 compartments of length l_j and diameter d_j . Membrane potential E_j calculated at the mid-point of each compartment. Internal conductances g_j are taken between the mid-points of the j -th and the $(j+1)$ -th compartment. Current is injected into the soma. The value I used in the figures is the absolute value I_m divided by the membrane area $\pi d l$ of the soma. E_R is the resting potential.

speed of computation for each compartment is proportional to the number of pores between approximately 1000 and 70000 pores, constant for larger pore numbers (Skauzen & Walloe 1979).

It is, however, felt that the simplification used here is an acceptable approximation. The compartments on the "input" side of the initial segment including the cell body have such large pore numbers that the fluctuations of the membrane potential will here be much smaller than in the initial segment and therefore of much less importance. The compartments on the output side of the initial segment represent the axon and have little effect upon the generation of the action potential in any case. It is expected that the effects of finite pore numbers in the model would have been somewhat enhanced if all compartments had been described by the stochastic model. The results presented here can therefore be considered as lower limits for these effects.

Another source of error is the division of the nerve cell into rather few compartments (usually 5). This was tested by a model where all compartments were governed by the H-H equations (no stochastic compartment).

The initiation of an action potential in the cell body and its time course in all the five compartments were calculated for different time step lengths Δt in the numerical solution, and for the three geometrical configurations used in most of the stochastic calculation (see Table 1). As an example of this Fig. 2 shows the membrane potential as a function of time in the third compartment for step lengths $\Delta t = 0.1$ ms and 0.05 ms (continuous lines). This can be compared against the broken line which shows the potential for the same configuration but with fifteen segments and $\Delta t = 0.005$ ms. The injected current (I in the cell body) is in all cases shown here $10 \mu A/cm$. Each of the 5 segments in the first case is now divided in 3 segments. Any further reduction of Δt did not change the result more than the line width in the figure. In all the three cases shown the curve for 5 segments and $\Delta t = 0.05$ ms is very close to the broken curve. The error introduced in the time (the time delay of the action potential) is always less than 0.1 ms for the value of the injected current used here. The time delay is larger near the current threshold for firing, but in all cases it corresponds to a variation in the injected current of less than 5% (A small

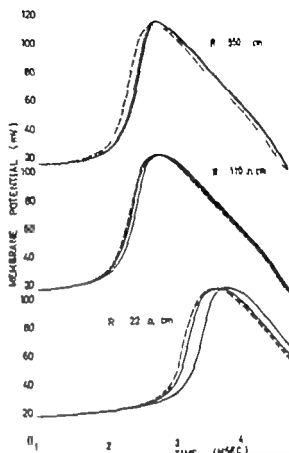


Fig. 2. Membrane potential at the mid-point of the last segment as a function of time for a non-stochastic calculation of model B for different values of R_m : 170 and 350 Ωcm . In each case the potential was calculated for 1 compartment and a step length $\Delta t = 0.005$ ms (broken curve) and for 5 compartments (unbroken curve) as $\Delta t = 0.1$ ms and 0.05 ms. The curve for $\Delta t = 0.05$ ms is closest to the broken curve.

decrease of the current of this amount would give the same time delay.) It is felt that this is accurate enough for our purposes. The segments in the nerve cell model and a time step length $\Delta t = 0.05$ ms are thus sufficiently accurate in the non-stochastic numerical calculation. The value of Δt was used as the maximum step length Δt_{max} in the stochastic calculation. It was tested and found sufficiently accurate here also (Skauzen & Walloe 1979).

In the stochastic compartment the membrane potential E_j is given by a set of equations similar to the ones describing the stochastic model presented before for a space-clamped nerve membrane. In all the other compartment the membrane potential E_j is given by the H-H equation. For all the compartments including the stochastic one these equations describe the membrane potential E_j as a function of time t and the injected current I :

$$E_j = E_j(t, I, N, S)$$

$$E_j = E_j(t, I, j, s)$$

(1)

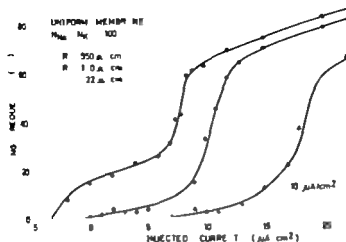


Fig. 5. Mean firing frequency as function of injected current for different values of R , for model B in the case of uniform membrane and $N_M = N = 100$. The curve for $R = 22 \Omega \text{ cm}$ differs for $10 \mu\text{A}/\text{cm}^2$ less than true value.

lead to a change in the geometrical configuration of the nerve cell.

The basis for the numerical solution of the equations in the stochastic compartment is the method termed *method B* in previous paper (Skaggen & Walløe 1979). The method is tested and found sufficiently accurate for a maximum step length Δt_{max} of 0.05 ms, which is also used here. The step length Δt is generated by stochastic process. This method (it is the time interval between recordings of the membrane conductances, limited open and closed) it can have any value between 0 and Δt_{max} . It is the step length used for all the other compartments.

For the stochastic compartment the time interval Δt is divided in two, in order to include the effects of the reset τ_r in Eq. (1). In the first half of the interval the reset E_r and E_{res} in Eq. (3) at the beginning of the interval are used. In the latter half the values at the end of the interval are used.

For the non-stochastic compartments the basis for the numerical solution is a faster version of the method used by Hodgkin & Huxley in the first solution of their equations (Hodgkin & Huxley 1952). This was found to be the best of a number of methods tested, and in addition an extremely stable one. Values of E_i are first predicted by predictor formulae. These values are then used in corrector formulae to predict new and better values of E_i . This is repeated once. Briefly stated, the whole numerical calculation is as follows:

We are at time t_n with the values of all parameters known. These are used by O. W. to go on to find the values of all parameters at time $t_{n+1} = t_n + \Delta t$. These are all indexed by 1.

1. By the drawing procedure described before (Skaggen & Walløe 1979), draw the time Δt to the next change of conductances in the stochastic compartment.

2. If $\Delta t > \Delta t_{\text{max}}$, set $\Delta t = \Delta t_{\text{max}}$ and skip numbers 11 and 12.

3. Predict values of E_j at time $t_{n+1} = t_n + \Delta t$: $E_j = E_{j0} + (dE_j/dt)\Delta t$ for $j = s$.

4. Predict a value of E_{res} : $E_{\text{res}} = EE_{\text{res}} + (E_{\text{res}} - EE_{\text{res}}) \exp(-[G/C_s]\Delta t)$, where $G = g_{\text{Na}} + g_{\text{K}} + g_{\text{A}} + g_{\text{leak}}$, $EE_{\text{res}} = (g_{\text{Na}}E_{\text{Na}} + g_{\text{K}}E_{\text{K}} + g_{\text{A}}E_{\text{A}} + g_{\text{leak}}E_{\text{leak}})/(g_{\text{Na}} + g_{\text{K}} + g_{\text{A}} + g_{\text{leak}})$ and g_{Na} , g_{K} , g_{A} and g_{leak} are the sodium and potassium conductances of the stochastic membrane and E_{Na} , E_{K} and E_{leak} are the equilibrium potentials for sodium, potassium and for the leakage conductance G_{leak} . C is the membrane capacitance.

5. Values of the α and β parameters in the H-H equations are calculated for all compartments using the values of E_i .

6. Values of the h and n parameters in the H-H equations are calculated by the corrector formulae.

$$h_{n+1} = (2C_s E_{\text{res}} \left(\frac{dh_n}{dt} + \alpha_{n+1} \right) / K' (1 - \alpha_{n+1}) \Delta t),$$

and correspondingly for m and n .

7. The value E_{res} at the middle of the interval Δt is calculated.

$$E_{\text{res}} = EE_{\text{res}} + (E_{\text{res}} - EE_{\text{res}}) \exp(-[G/C_s]\Delta t)$$

8. Calculate new values of E_i ($j = s$) by the corrector formulae:

$$E_{\text{res}} = (2C_s E_{\text{res}} + (C_s \frac{dE_{\text{res}}}{dt} + g_{\text{Na}} h_{n+1} m_{n+1}^3 E_{\text{Na}} + g_{\text{K}} n_{n+1}^4 E_{\text{K}} + g_{\text{A}} E_{\text{A}} + g_{\text{leak}} E_{\text{leak}}) \Delta t) / (C_s + (g_{\text{Na}} h_{n+1} m_{n+1}^3 + g_{\text{K}} n_{n+1}^4 + g_{\text{A}} + g_{\text{leak}}) \Delta t).$$

9. Calculate new value of E_{res} .

$$E_{\text{res}} = EE_{\text{res}} + (E_{\text{res}} - EE_{\text{res}}) \exp(-[G/C_s]\Delta t), \text{ where } EE_{\text{res}} = (g_{\text{Na}} E_{\text{Na}} + g_{\text{K}} E_{\text{K}} + g_{\text{A}} E_{\text{A}} + g_{\text{leak}} E_{\text{leak}}) / (g_{\text{Na}} + g_{\text{K}} + g_{\text{A}} + g_{\text{leak}}).$$

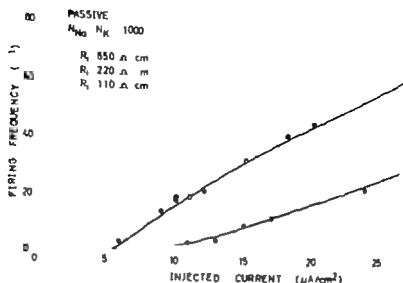


Fig. 4. Mean firing frequency as function of injected current for different values of R_i for model B in the case of a passive soma and $N = N_K = 1000$.

The same model equations can however be obtained from several different sets of parameters for the nerve cell. Assume that all the rate constants are multiplied by ϕ , all the maximal membrane conductances are multiplied by η , the specific internal resistance is multiplied by w , all the diameters are multiplied by r , all the segment lengths are multiplied by q and the conductances of single pores are multiplied by u . The basic equations for the model can then be written as

$$C \frac{dE}{dt} = -\frac{\eta}{q} \left(g_{Na}(E - E_{Na}) + g_K(E - E_K) + g_L(E - E_L) \right) + k_1 \frac{1}{\eta} + \frac{r}{\eta w q^2 A_j} \quad (3)$$

where

$$r = \phi l, \quad A_j = \pi r d, \quad l_j$$

$$g_{Na} = g_{Na}(\tau = \beta_1 S), \quad x = h \text{ or } m$$

$$g_K = g_K(\alpha = \beta_2 S)$$

$$N = \frac{\eta r q}{u} N_0$$

and where C , g_{Na} , g_K and g_L are the specific membrane capacitance and the specific sodium, potassium and leakage membrane conductances respectively. E_{Na} , E_K and E_L are the equilibrium potential for sodium, potassium and the leakage ions respectively. The currents and l_{Na} are defined in Eq. (2). g_{Na} and g_K are functions of the transformed time τ , the rate constants α and β , which are functions of the potential E only and of a stochastic process S for $j = a$. N and N_0 indicate the number of pores in the stochastic compartment before and after the transformation.

We see that the equations are left formally unchanged if $\phi = \eta$, $r = \eta w q^2$ and $u = \eta r q$. The injected current depends upon η but as it is a free variable it can be adjusted

correspondingly. Instead of the real time t the transformed time $\tau = t/\phi$ is used, which means that the frequencies calculated and presented here must be multiplied by ϕ .

In a former paper it was shown that ϕ probably was roughly equal to η over a wide range of values of (Skaugen 1979). This will be assumed to be the case here. The first condition $\phi = \eta$ is then satisfied and the conditions for formally unchanged equations can be written as

$$r^2 = \frac{w}{\eta} l^2, \quad q = \frac{l}{w \eta}, \quad \phi = \eta$$

If the conductances for single pores are assumed constant the parameter u is constant and r , q and ϕ depend upon w and η only. In a given nerve cell model the pore diameter q and the specific internal resistance R_i can thus be changed to arbitrary new values ηq and $w R_i$, thus changing the firing behaviour. If the diameters and lengths of the model compartments are multiplied by the factors r and q given by Eq. (4) in the same way the assumed conductances for single pores can be changed. Summarized, a single specified model thus covers a wide range of possible models.

Equation (4) also shows that a change in one parameter corresponds to changes in other parameters. I found it convenient to investigate different cell models by changing the specific internal resistance R_i from $2 \Omega \cdot \text{cm}$ to $550 \Omega \cdot \text{cm}$. If $R_i = 110 \Omega \cdot \text{cm}$ is taken as the standard value, w is then changed from 0.1 to 5. This corresponds to keeping R_i constant at 1 standard value while the compartment diameters and lengths are changed by factors ranging from $r = 1/\sqrt{0.2} = 1.71$ and $q = \sqrt{0.2} = 0.447$ to $r = 1/\sqrt{5} = 0.447$ and $q = \sqrt{5} = 2.24$. This latter change of the cell geometry is more interesting, but also more complicated to keep track of. We may thus take the simple change of the internal specific resistance as a short-

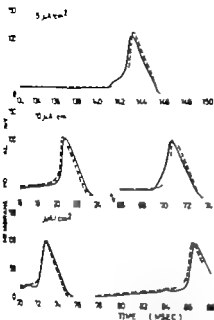


Fig. 7. Membrane potential as function of time in soma (solid curve) and in initial segment (unbroken curve) for different values of the injected current. Note break in middle curve ($10 \mu\text{A}/\text{cm}^2$).

then be multiplied by ϕ and η as explained before. The sodium and potassium pore densities were assumed equal, and the total numbers N_{Na} and N_{K} of pores are given the values $N_{\text{Na}} = N_{\text{K}} = 100$ and $N_{\text{K}} \sqrt{1000}$.

Figs 3 and 4 show the mean firing frequency f as a function of the injected current I when $N_{\text{Na}} = N_{\text{K}} \sqrt{1000}$. For each figure three different values of the specific internal resistance R_i are used. According to the equivalent transformations described in Eq. (4) this corresponds to three different geometrical configurations with $R_i = 110 \Omega\text{-cm}$ as shown by Table 1.

The case of uniform membrane is shown by Fig. 3 that is, the maximal specific membrane conductances are equal in all the different compartments representing the cell body, the initial segment, and the axon. The frequency-current relationship is very close to the relationship obtained in a non-stochastic nerve cell model where the potential in all compartments is calculated by the original H-H equations. In this case the mean firing frequency f increases very rapidly from zero to approximately 55 s when the injected current I is increased. This happens at the threshold current for

firing. The slight increase of the firing frequency f above zero at somewhat smaller values of the current which is shown by Fig. 3 is thus absent in a non-stochastic model. But in spite of this difference the overall effects of a finite pore number upon the firing behaviour are very small in this case.

This is quite different in the case of a cell body (or soma) with a passive membrane that is, in the soma there is only a leakage conductance which is constant and independent of the membrane potential. As shown by Fig. 4 there is now a much more linear frequency-current relationship. This is not the case in the corresponding non-stochastic model where the frequency increases rapidly from zero to a high value when I is increased above a certain threshold value. This corresponds more to the curves shown by Fig. 3.

The difference between the two cases shown by Figs 3 and 4 is not difficult to understand. For uniform membrane the soma can fire, but the initial segment has a lower threshold for firing than the soma due to the stochastic fluctuations of the membrane conductances (Skauzen & Walloe 1979). On the other hand the current is injected in the soma, and the mean membrane potential therefore is somewhat higher there than in the initial segment. As the injected current I is increased the (stochastic) initial segment starts to fire, but for slightly larger values of I the threshold current for the non-stochastic soma is reached and the soma starts to fire at a high frequency as in the original H-H equations (Hodgkin & Huxley 1952). In the uniform membrane model the threshold current is $6.3 \mu\text{A}/\text{cm}^2$ but here the soma is loaded by the initial segment and the axon, and part of the injected current goes to these compartments. As the internal resistance is decreased the load is increased, and the threshold current for firing is increased as shown by Fig. 3.

When the soma is passive however it cannot generate an action potential and the firing behaviour is more determined by the properties of the stochastic initial segment. For not too large pore numbers ($\leq 20,000$) the frequency-current relationship for the stochastic segment is distinctly more linear than for the original H-H equations, as shown before (Skauzen & Walloe 1979; Skauzen 1980). This gives the fairly linear frequency-current relationship for the whole nerve cell. Since the membrane potential of the initial segment is only indirectly affected by the injected current (via the

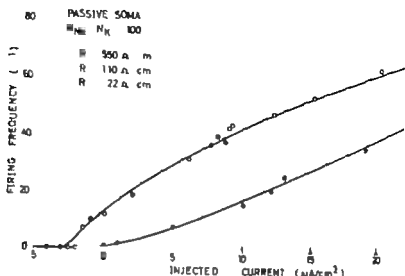


Fig. 6 Mean firing frequency as function of injected current for different values of R_i for model B in the case of a passive soma and $N_{Na} = N = 100$

10 Repeat step 5 6 7 8 and 9

11 By the drawing procedure described before (Skaugen & Walloe 1979) draw what the changes in the membrane conductances g_m and g_a of the stochastic compartment are. Change these conductances accordingly.

1 We are now at time t with the values of all parameters known. Go to step 1 and repeat the procedure.

In addition to this the potential of the s -th compartment will be calculated by the H-H equations as in the other compartments during an action potential as described earlier (Skaugen & Walloe 1979).

In the actual program every care was taken to avoid unnecessary operations in the computation loop shown here. The quantities used in the actual program were therefore combinations of the symbol shown here and the expressions were considerably simpler. The greatest saving in computer time was obtained in step 5. Before the start of the simulation the values of the α and β parameters in the H-H equations were calculated for E equal to $E_h - 1$ mV. In steps of 1 mV until $E_{Na} + 1$ mV and then stored in an array. In step 5 the α and β values are found by direct linear interpolation between the values stored in this array.

The results of the simulations depend upon a stochastic process (employing two quasi-random number generators) and for the same parameters different simulations (starting with different seeds in the random generator) will give values distributed around a mean value. The standard deviations of the results were calculated but not shown in the figures to avoid cluttering. The scatter of the simulation results around the curves will however indicate the amount of uncertainty. The curves are drawn by hand as the best fit to the simulation results.

The models were usually simulated for one second of model time but some times for two seconds at low firing frequencies. This time was too short to obtain reliable

estimates of the standard deviation of the interspike intervals, the burst factor etc. A was done for the membrane models described earlier (Skaugen & Walloe 1979, Skaugen 1980).

It should be noted that in a non-stochastic nerve cell model based only upon the original H-H equations, the membrane potential will always be highest at the point of current injection when there is no firing. In the model shown in Fig. 1 a current will flow axially from the soma towards the axon as the latter is closer to the resting potential. A depolarizing injected current is assumed. As the current flows towards lower potentials, the potential must decrease from the soma toward the initial segment and the axon. In the case of a uniform membrane the threshold for firing will therefore be reached first in the soma if the injected current is increased. The spike will therefore always start in the soma. This is generally not the case in the stochastic models described here. In a non-stochastic model the spike will start in the beginning of the axon only if the threshold for firing is somewhat lower than in the soma. The axon membrane must then have some properties different from those of the soma membrane, for example a shift in the potassium dependence for sodium conductance or a higher density of sodium conducting pores.

RESULTS

Table 1 shows examples of the nerve cell models used here for a few different values of ϕ , assuming $u=1$ and a sodium pore density of 500 pores/ μm^2 . In squid. All the actual simulations were performed with $\phi=1$ such that the value of η shown in the figures is the value of $\eta = \eta/\phi$ if we use the results for $\phi=1$. The frequencies and currents shown must

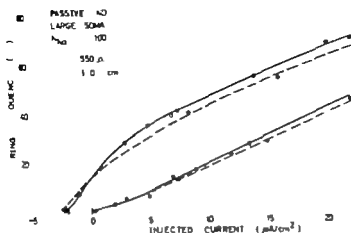


Fig. 9. Mean firing frequency as function of injected current for different sizes of R_i in the case of passive soma for large soma (unbroken curve model E) and for normal soma (broken curve model B). $N_{\text{ax}} = N = 100$.

$1 \mu\text{A}/\text{cm}^2$ the spike is in most cases generated first in the initial segment and then spreads up to the soma and down the axon as shown for $i = 5 \mu\text{A}/\text{cm}^2$ in the figure. It should be noted that in a nerve model based wholly upon the original H-H equations the spike will always start at the soma, at the point of current injection, in the case of a uniform membrane.

On the steep middle part of the curve from approximately 8 to $13 \mu\text{A}/\text{cm}^2$ the spike may arise first in either the soma or the initial segment as shown by the two neighbouring action potentials for $i = 10 \mu\text{A}/\text{cm}^2$. On the upper part of the frequency-current relationship the spike is in most cases generated first in the soma as shown for $i = 15 \mu\text{A}/\text{cm}^2$. The soma is now fired directly by the injected current and the potential in the initial segment just follows.

For a real nerve cell where the excitability of the membrane probably decreases from the initial segment toward the dendrites, the tendency for the spike to start at the initial segment will be stronger. The cell then lies closer to the case of passive soma where the spike always is generated in the initial segment.

In the following figures some parameters are changed to obtain model which cannot be obtained by the equivalent transformations described here. Figs. 8 and 9 show the frequency-current relationship when only the soma is increased in size (con-

tinuous line) as compared to the former case (broken line). For the uniform case shown by Fig. 8 the lower part of the two curves where the spike is generated in the initial segment are almost identical. But the larger soma is relatively less loaded by the initial segment and the axon and its firing behaviour is less affected and closer to that of the isolated space-clamped membrane with its steep increase of the frequency around the current threshold for firing. This difference is clearly seen in the upper part of the curve.

For the case of the passive soma shown by Fig. 9 the difference between the large soma (continuous line) and the former case (broken line) is very small. The larger soma is able to maintain a slightly larger membrane potential at a certain value of the injected current because it is relatively less loaded. This gives a slightly larger value of the axial current reaching the initial segment where the firing is started. But the resulting increase in the firing frequency is hardly significant. For the passive soma the size of the soma thus seems to influence the firing behaviour very little when the cell body is not too small.

The effects of reducing the maximal membrane conductances without changing the rate constants are shown by Figs. 10 and 11. For a constant pore conductance ($\gamma = 1$) this corresponds to a similar reduction of the pore densities. The condition $\phi = \eta$ in Eq. (4) now no longer holds true and the equiv-

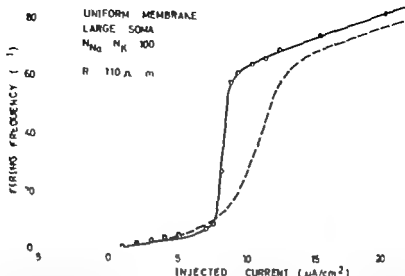


Fig. 8. Mean firing frequency as function of injected current for $R_i = 110 \Omega\text{-cm}$ in the case of a uniform membrane for a large soma (unbroken curve: model E) and for a normal soma (broken curve: model B). $N_{Na} = N_K = 100$.

soma) and also due to the load of the axon the effective current exciting the initial segment is considerably smaller than the injected current i shown in the figures. The firing frequency therefore increases slower when i is increased than found for the uniform membrane. The special geometry of the nerve cell thus helps to make the frequency-current relationship more linear over a larger current range. This is clearly seen by comparing Fig. 4 and the curve for N_{Na} or $N_K = 1000$ in Fig. 4 and 5 in an earlier paper (Skaugen & Walloe 1979). But this depends upon the stochastic properties of the initial segment. In a non-stochastic nerve cell model the initial segment has a sharp threshold for firing and this will give the sudden increase in the firing frequency so characteristic for the H-H equations.

As the specific internal resistance R_i is decreased the initial segment is more heavily loaded by the soma and the axon and at a certain point an action potential can no longer be generated because the current lost axially is too large. This happens for $R_i = 110 \Omega\text{-cm}$ in Fig. 4.

Figs. 5 and 6 show the firing frequency as a function of the injected current for the smaller pore numbers $N_{Na} = N_K = 100$. In the case of a uniform membrane the threshold for firing in the initial segment is now sufficiently small to affect the firing behaviour considerably. The sharp threshold for firing which was seen for 1000 pores is now seen as the steep middle part of the curve while the lower

slowly rising part of the curve has become much larger. This gives the frequency-current relationship a distinct sigmoid shape. The lower part of the curve can be reasonably well approximated by two straight lines: one with a small slope for small values of the injected current and one with a larger slope for larger values. This resembles the primary and secondary firing range observed in some nerve cells (Kernell 1963) but other mechanisms have been found which can explain this (Kernell 1971).

For a passive membrane in the soma the firing behaviour for $N_{Na} = N_K = 100$ as shown in Fig. 6 is much more similar to the behaviour shown in Fig. 4 for larger pore numbers. The frequency-current relationship is fairly linear and its slope is small. The main difference between the curves shown by Figs. 4 and 6 is a current threshold for firing which is approximately $10 \mu\text{A}/\text{cm}^2$ smaller in Fig. 6 than in Fig. 4. There is also a firing response for smaller values of the specific internal resistance R_i for the smallest pore numbers.

The action potential is always initiated in the initial segment when the soma membrane is passive because the soma then cannot fire. The situation is more complex in the case of a uniform membrane. Fig. 7 shows typical examples of action potentials as registered in the initial segment (continuous line) and in the soma (broken line) in the standard model for $R_i = 110 \Omega\text{-cm}$ shown in Fig. 8. On the slowly rising lower part of the curve to approximate

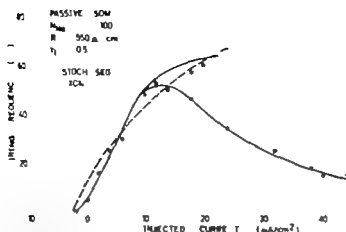


Fig. 11. Mean firing frequency—reaction of injected current for $R_i = 550 \Omega\text{-cm}$ and for $\eta = 0.9$ (solid line) and $\eta = 1.0$ (broken line). Open and filled circles represent firing in the total segment and in the axon, respectively. $N_{\text{axon}} = N = 100$. Passive soma.

using H-H equations the frequency-current relationship was quite linear over a considerable range of the injected current in the case of a passive soma. In fact, it was more linear than for the simpler membrane model discussed before (Skaugen & Walløe 1979; Skaugen 1980). It is thus seen that the geometry of the nerve cell can be important for its firing behaviour.

The nerve cell models were not simulated with pore numbers larger than $N_{\text{axon}} = N = 1000$ in the total segment. But the results obtained for the local membrane showed that a significantly more linear frequency-current relationship than for the H-H equations was obtained for pore numbers up to 20 000 (Skaugen & Walløe 1979). Since the geometry of the nerve cell increases this linearity in the case of a passive soma we may expect significant differences from a non-stochastic model at least up to this pore number. As previously noted the simulated model time was too short to obtain reliable estimates of the variability of the interspike intervals, but some general comment can be made. In the case of a uniform membrane the firing was clearly very regular for frequencies higher than 50–60 Hz, which corresponds to the threshold for firing in the Hodgkin-Huxley model. For lower frequencies and in cases of passive soma the standard deviation ΔT of the interspike intervals was significantly different from the relation found in the membrane model described in an ear-

lier paper (Skaugen & Walløe 1979 (their Fig. 8)) where $\Delta T = 0.8/f$ for low firing frequencies in models with pore numbers less than 10 000. The discharge will in these cases be quite irregular. For instance at a mean firing frequency f of 10 s the interspike intervals T would mainly lie in the range $T = 1/f - \Delta T = 20$ ms to $T = 1/f + \Delta T = 180$ ms.

It thus seems that for neurones firing quite regularly at a low frequency the stochastic processes described here would not be of any major importance for the observed frequency-current relationship. A low regular firing frequency is then probably due to a nerve membrane with somewhat different properties from those described by the Hodgkin-Huxley model as for instance in the membrane model suggested by Connor & Stevens (1971) where low firing frequencies could be generated.

The burst firing reported for the stochastic membrane model was not investigated here mainly because it was connected with higher pore numbers than used here but also because the model time used in the simulation (1 s) was too short to investigate finer details in the distribution of firings in time. From the results obtained however no tendency to burst firing could be seen for the case of passive soma. For the case of a uniform membrane there was a tendency to burst firing for small frequencies (at the foot of the curves in Fig. 3) but only for the largest pore numbers used.

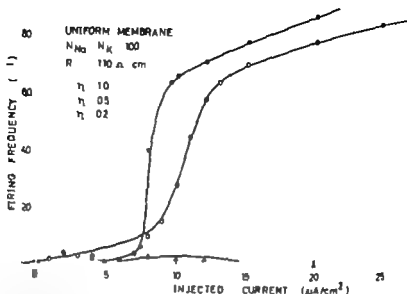


Fig. 10. Mean firing frequency as function of injected current for $R_i = 110 \Omega\text{-cm}$ and for different values of the maximal relative membrane conductances η . $N_{Na} = N_K = 100$. Uniform membrane.

alent transformation described here cannot be used.

In the case of the uniform membrane the frequency-current relationship is much steeper for $\eta = 0.5$ and for $\eta = 0$ the cell hardly fires at all. The membrane currents become too small compared to the membrane capacitance and the axial currents to sustain firing when η is reduced first in the initial segment as seen by the reduction of the firing at the lower part of the curve for $\eta = 0.5$ and then for the soma also as seen for $\eta = 0.2$.

In the case of a passive soma the reduction of η from 1 (broken line) to 0.5 (continuous line) does not change the frequency-current relationship much for $i \leq 10 \mu\text{A}/\text{cm}^2$ as seen from Fig. 11. For larger values of the injected current i the firing frequency f decreases when i is increased (filled circles). In the initial segment however f does not decrease (open circles). The firing frequency is taken as the number of spikes generated in one second. A spike is defined to have occurred if a certain threshold value of the membrane potential is reached; in this case 50 mV above the potassium equilibrium potential E_K is used. As the filled circles show the firing frequency in the axon we see from Fig. 11 that an increasing number of spikes is not transmitted from the initial segment and down the axon when i is increased. This effect is not of course restricted to values of η less than one but it was not seen in the other cases studied here for the current ranges shown in the figures. It was

however observed for $\eta = 1$ and $R_i = 110 \Omega\text{-cm}$ shown by the lower curve in Fig. 6 when i was increased above $5 \mu\text{A}/\text{cm}^2$. For smaller values of i all the spikes were transmitted as for $\eta = 0.5$ when i is less than $10 \mu\text{A}/\text{cm}^2$.

When $\eta = 0$ the cell did not fire at all. The reason is the relatively heavier loading of the spike generating compartment by the axial current and membrane resistance as for the case of the uniform membrane.

CONCLUSION AND DISCUSSION

The results presented here show that the existence of a finite number of two-state pores can significantly influence the firing behaviour of nerve cells. The number of pores in the initial segment is an important parameter. For the numbers used here 100 and 1000 the effects upon the firing behaviour were highly significant for the case of a passive soma but only for $N_{Na} = N_K = 100$ for the case of a uniform membrane with the same pore density all over the cell. In this latter case the firing behaviour for $N_{Na} = N_K = 1000$ was very close to that of a non-stochastic model. This model predicts a non-linear frequency-current relationship quite similar to the curves shown by Fig. 3 both in the case of a passive soma and of a uniform membrane.

In spite of the highly non-linear behaviour of the

number of sodium and potassium pores. As shown earlier for the uniform membrane the frequency-current relationship is approximately the same for the two different pore systems taken separately (for pore numbers ≥ 1000), and their combined effect gives only a slightly higher firing frequency (Skauzen & Walløe 1979). For smaller pore numbers the firing behaviour is relatively more dominated by the potassium pore system. As the existence of a two-state sodium pore system is fairly certain (Coeb & al 1976) while this is not known for the potassium pore system where the pore densities also are very uncertain, we could have calculated a maximum effect of a finite pore number by assuming an infinite number of potassium pores. This situation would be approximated in any case when the membrane electrical noise is dominated by fluctuations of the sodium conductance as would be the case if either the potassium pore density is larger than the sodium pore density or the potassium pores do not operate on an on-off basis. The overall firing frequency would then be somewhat smaller than shown in the figures for $N_{Na}/N_K = 100$ and only slightly smaller than shown for $N_{Na}/N_K = 1000$. See also the discussion at the end of the former Papers (Skauzen 1980).

In the former papers effects of other types of electrical noise in the nerve membrane than conductance fluctuations were also briefly discussed. It is pointed out there any additional noise would increase the fluctuations of the membrane potential and thus increase the effects studied here. The effects of gating currents were studied in more detail and it is shown that these effects were proportional to the relative constants ϕ . Even if gating currents are important in large nerve fibres it seems that in the small nerve cells of interest here the rate constants are much smaller than in large nerve fibres, the effects of gating currents would be small.

For the small values of ϕ shown in Table 1 the time course of the action potential in small nerve cells may seem to be excessively slow. But it should be noted that the results are obtained for a temperature of 6 °C, and at higher temperatures all processes in the nerve membrane will go faster.

The pore density and the dimensions of the initial segment determine the axial resistance and the number of pores in this region, and these parameters were found important for the firing behaviour. By means of the equivalent transforma-

tions given by Eq. (4) the models presented here can be used for any pore density. Fig. 1 shows the relation between pore density ρ and the diameter d_s of the initial segment for models A, B and C in Table 1 under these transformations when $R_i = 110 \Omega\text{-cm}$. The conductances of single pores are assumed equal to those in squid ($u=1$). The relations are shown both for $N_{Na}=N_K=1000$ (continuous lines) and for $N_{Na}=N_K=100$ (broken lines). Along these lines the firing behaviour does not change except that the mean firing frequencies and the injected current shown in the figures are multiplied by $\rho/500 \text{ pores}/\mu\text{m}^2$.

The model corresponding to the curve for $R_i = 20 \Omega\text{-cm}$ in Fig. 4 lies between curves B and C in Fig. 12. This will be the uppermost curve where a linear frequency-current relationship was found in the simulations. But from results obtained from the membrane model we suspect model C with for instance 10000 pores to give a frequency-current relationship significantly different from the non-stochastic case. This model will be represented by a curve slightly above the continuous curve marked B in Fig. 1.

The circles represent measured pore densities in different nerve fibres where the range in fibre diameters is indicated by the vertical bars (Colquhoun et al. 1977). The dotted curve is an approximate fit to these data with the pore density for the squid giant axon included ($\rho_{Na} = 500 \text{ pores}/\mu\text{m}^2$, $d = 500 \mu\text{m}$ to $900 \mu\text{m}$ (Keynes & Rojas 1974)). This lies outside the region shown by the figure.

It is seen that all the types of fibres shown here include diameters where the effects due to a finite pore number are significant. The garfish olfactory nerve corresponds for instance to the curve for $R_i = 550 \Omega\text{-cm}$ in Fig. 5. Even if the pore densities at the site for spike generation are higher than in the axon, the figure indicates that some effects are expected for small fibre diameters. Even for pore densities around $100 \text{ pores}/\mu\text{m}^2$ the diameter need not be smaller than $0.1 \mu\text{m}$. In general we may conclude that if the diameter at the site for spike generation is less than $0.5 \mu\text{m}$ to $1 \mu\text{m}$, and the length larger than $0.5 \mu\text{m}$, space constants effects due to a finite pore number may be suspected.

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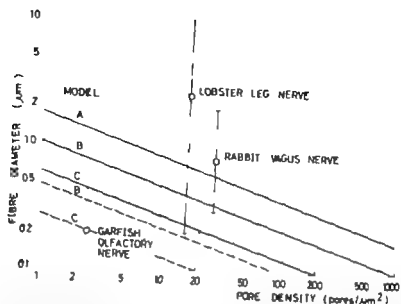


Fig. 12 Relation between the diameter of the initial segment and its pore density for some of the cell models used under the equivalent transformations. Continuous lines for $N_i = N_e = 1000$ and broken lines for $N_i = N_e = 100$. Circles show measured sodium pore densities in different nerve fibres with range of fibre diameters indicated by vertical bars. The length L of the initial segment in units of the space constant λ is 0.18, 0.41 and 0.91 respectively for the continuous lines A, B and C. Dotted curve is a fit to experimental data, see text.

$N_i = N_e = 1000$. Whether burst firing would occur for larger pore numbers for the passive soma remains to be investigated, but the behaviour of the membrane model strongly suggests that it would be seen at sufficiently large numbers (> 10000).

Small nerve fibres seem to have smaller pore densities than larger fibres (Jack 1975). This does not change the model equations if the rate constants are reduced correspondingly. But a reduction of the pore density relative to the rate constants seems to be very detrimental for the cell's ability to fire, as shown by Figs 10 and 11. This is in contrast to the membrane model where a reduction of pore densities alone (not changing rate "constants") down to 0.2 did not stop the firing (Skaugen 1980). This difference is mainly due to the axial currents which are present in the cell model. These depend upon the internal resistance and are not changed when the pore densities are changed. The initial segment is therefore more and more effectively short-circuited to the cell body when the pore densities are reduced. This can be counterbalanced by a corresponding increase of the internal resistance which corresponds to a longer and thinner nerve cell when R_i is kept constant. But it is felt that these difficulties with a reduction of pore densities relative to the rate constant (ρ/ϕ) do strengthen the

results of the discussion in an earlier paper when it was shown that if the H-H equations are used to describe impulse conduction in small or myelinated nerve fibres the few available experimental data suggest that ρ/ϕ is constant or even increases when the fibre diameter decreases (Skaugen 1980).

It is now a question how well the nerve cell models presented here represent real nerve cells. As pointed out before nerve cells seem to have pore densities which decrease from the initial segment and towards the dendrites. They would thus lie between the two cases investigated here. It is expected that the effect of reducing the pore density in the soma is to move the steep part of the frequency-current relationship in the unfused membrane case towards larger currents, because this part is due to firing in the soma. For a sufficiently small pore density in the soma this relationship would therefore be almost identical to that of the passive soma for reasonable values of the injected current.

We have here assumed an equal density of sodium and potassium conducting pores, and that both pore systems operate on an on-off basis. The firing behaviour of the model is not, however, very sensitive to the numerical ratio between the

Decreased release of somatostatin into the portal vein following electrical vagal stimulation in the cat

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Electrical vagal stimulations were performed in anesthetized cats, in which the spleen and the intestine below the duodenum had been removed. The cats were provided with acute atrial perfuses which were perfused during the experiments. Insulin, glucagon, gastrin and somatostatin were measured in portal vein blood (consisting of blood from the stomach and the pancreas). The atrial perfusates were analyzed for gastrin and somatostatin. Electrical vagal stimulation increased the release of insulin and glucagon into the portal vein. Gastrin was elevated in atrial perfusates and portal vein blood whereas somatostatin increased in the atrial perfusates, but was reduced in portal vein blood. We assume that atrial somatostatin, like gastrin, is released into the blood. The finding that portal vein somatostatin was decreased following vagal stimulation indicates that the release of somatostatin from another large source of somatostatin, presumably the fundic region of the stomach or the pancreas, was decreased by vagal activation.

Key words: Vagal nerve, insulin, glucagon, gastrin and somatostatin.

Several peptide hormones are located in the endocrine cells of the pancreas and of the mucosa of the gastrointestinal tract: e.g. insulin, glucagon and somatostatin containing cells in the pancreas, gastrin containing G-cells in the atrial mucosa and somatostatin containing D-cells along the entire GI tract. The release of these peptides is partly under nervous control. Accordingly, in dogs the output of insulin, glucagon and gastrin increases after sham feeding or electrical vagal stimulation (Frohman, Ezdinly & Javid 1967; Kaneto, Kosaka & Nakano 1967; Kaneto, Miki & Kosaka 1974; Lanciault et al. 1973; Becker, Reeder & Thompson 1974; Smith et al. 1975; Nilsson et al. 1972; Tepperman, Walsh & Pinkus 1972; Nilsson & Uvnäs-Wallensten 1974; Uvnäs & Uvnäs-Wallensten 1977). In cats, electrical vagal stimulation enhances the release of insulin, glucagon and gastrin into the portal vein (Uvnäs-Wallensten & Nilsson 1977; Uvnäs-Wallensten & Nilsson 1976) and also the release of gastrin and somatostatin into atrial perfusates (Uvnäs-Wallensten 1977; Uvnäs-Wallensten, Efendic & Luft 1977).

In the present study we have investigated the effect of electrical vagal stimulation on somatostatin release in cats *in vivo*. Somatostatin was determined in the portal blood and in the atrial perfusate.

MATERIAL AND METHODS

Experimental procedures

The cats were anesthetized with chloralose and urethane (90 and 100 mg/kg). The trachea was cannulated and the animals breathed spontaneously. Blood pressure was continuously measured in femoral artery.

The vagal nerves were isolated and cut in the cervical region. The distal ends were stimulated electrically at 10 V, 2 ms duration and 5-10 Hz during 5 min.

To allow collection of portal blood the spleen and the

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| | |
|-------|-------|
| 30 | 40 |
| 1 000 | 1 200 |
| 1 400 | 800 |
| 1 800 | 1 400 |
| 200 | 400 |
| 250 | 600 |
| 300 | 650 |
| 0 | 100 |
| 0 | 90 |

Expts. produced by themselves, were used at a final dilution of 1:4000. Crossreactivity of the antibody was less than 0.1% with insulin, glucagon, substance P, LH, RH, vasopressin and oxytocin. The antigenic specificity of the antibodies has been determined using somatostatin analogues (Arimura et al. 1978). Phosphate buffer (0.4 M pH 7.4) containing 1% bovine serum albumin was used as diluent for all components in this RIA. The incubations were conducted for 48 h at 4°C. Separation of bound from free binder was accomplished with dextrane-coated charcoal (Arimura et al. 1973). Somatostatin in plasma was measured after acetone extraction (Arimura et al. 1978). 50–100% of synthetic somatostatin added to plasma was recovered after the full extraction procedure. Dilution of both the extracted plasma samples and antral perfusates demonstrated parallel binding to that of the synthetic standard. The sensitivity of the assay was 5 pg/ml (Eberhardt et al. 1979).

RESULTS

Hormone release into the portal vein

Somatostatin in portal vein blood was measured in 8 expts before and during electrical vagal stimulation. In 3 of the expts, the antrum was perfused with 0.1 M HCl (pH 1.2) and in 5 expts, with 0.15 M NaCl (pH 4.5). Before the stimulation somatostatin averaged 650 pg/ml (range 115–1500). It decreased within 5 min of stimulation (Table 1, Fig. 1) and again reached prestimulatory values within 10–20

min after its cessation. The same pattern was observed irrespective of whether the stimulations were performed with 5 or 10 Hz, or if the antrum was perfused with 0.15 M NaCl or 0.1 M HCl.

Portal vein insulin, glucagon and gastrin levels were measured in 2 of the expts (Fig. 1). During vagal stimulation the output of these hormones reached a peak within 10 min. The levels then decreased and reached the basal ones within 10–20 min.

Hormone release into the antral perfusate

During prestimulatory conditions considerable amounts of somatostatin were released into the antral lumen provided the perfusates had a low pH (exp. 1–3, Table 1). On the other hand when the antra were perfused with 0.15 M NaCl only small amounts of somatostatin were released in 2 out of 5 expts. (exp. 4 and 6, Table 1).

When the vagal nerves were stimulated, a clear increase in somatostatin output occurred in the 3 expts in which the antrum was perfused with 0.1 M HCl. During perfusion with NaCl a slight increase in somatostatin was observed in the 2 expts. in which measurable amounts of somatostatin were released under prestimulatory conditions (Table 1). A typical experiment, demonstrating the effect of vagal stimulation on the release of somatostatin and gastrin into the portal vein and into the antral lumen is shown in Fig. 2.

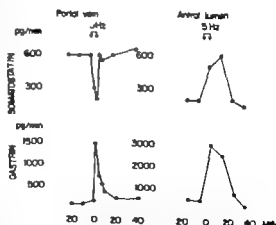


Fig. 2. Release of somatostatin and gastrin into the portal vein and antral lumen following electrical vagal stimulation (5 Hz). Note that somatostatin output into the portal vein decreases whereas slight increase is seen in antral perfusates (Expt. 6 from Table 1).

Table 1 Release of somatostatin into the portal vein (p) and into antral perfusates (a) following electrical vagal stimulation at 5 or 10 Hz during antral perfusion with 0.1 M HCl and 0.15 M NaCl

| Exp no | Exp conditions | Time in minutes | | | | | | | |
|--------|----------------|-----------------|--------|------|------|------|-----|------|------|
| | | -70 | -10 | 0 | 5 | 10 | 15 | 20 | 25 |
| 1 | 10 Hz HCl | p 1500 | a 1700 | 1500 | 1400 | 700 | 700 | 1000 | 1400 |
| 2 | 10 Hz HCl | p 600 | a 000 | 2000 | 800 | 700 | 350 | 1200 | 1000 |
| 3 | 10 Hz HCl | p 1700 | a 1500 | 1500 | 1400 | 1200 | 800 | 400 | |
| 4 | 10 Hz NaCl | p 400 | a 200 | 250 | 400 | 150 | 100 | 200 | |
| 5 | 10 Hz NaCl | p 600 | a 0 | 0 | 650 | 50 | 700 | 50 | 300 |
| 6 | 5 Hz NaCl | p 600 | a 700 | 700 | 600 | 400 | 700 | 600 | 350 |
| 7 | 5 Hz NaCl | p 150 | a 0 | 0 | 120 | 40 | 50 | 60 | 70 |
| 8 | 5 Hz NaCl | p 120 | a 0 | 0 | 110 | 10 | 70 | 30 | 100 |

Intestine below the duodenum were removed a catheter inserted into the blind end of the superior mesenteric vein and the portal vein tied close to the liver. Thus the portal vein conveyed blood from the stomach, the pancreas and the duodenum backwards out of the cat. Before entering the cat again the blood passed a drop-chamber for recording of the blood flow. The cats were heparinized to

avoid clotting of the blood. The preparation has been described in detail in a previous publication (Uvnäs-Wallensten, Uvnäs & Nilsson 1976).

Blood samples (5 ml) were collected at -20, -10, 0, 2, 5, 7, 10, 20 and 40 min. Trasylol (10%) was added to the samples that were immediately put on ice. After centrifugation plasma was taken off and frozen.

To allow determination of the intra-antral secretion of gastrin and somatostatin acute antral pouches were formed by tying ligatures around the pylorus and the antrum-corpus border. The antrum was then perfused with 0.1 M HCl or 0.15 M NaCl (38°C) at a rate of 1 ml/min. The perfusate entered the antrum via a catheter introduced into the pylorus and left it via a small cannula inserted into the antral wall. For further details of the method see Uvnäs-Wallensten (1977).

The antral perfusates were collected in tubes surrounded by ice during 10-min periods. pH was adjusted to 7 and the samples boiled for 10 min before being frozen. The rate of release into the circulation and into the antral lumen of the different peptides was calculated by multiplying hormone concentrations and portal plasma flow and the flow rate of the antral perfusate respectively.

Radioimmunoassay

Insulin was determined by a double-antibody radioimmunoassay (Hales & Randle 1963) using insulin reagent kits (Radiochemical Centre, Amersham). Glucagon was assayed using an antibody specific for pancreatic glucagon (30 K) (Agvilar, Parada, Essentiaut & Unger 1969). Gastrin was determined by the method described by Nilsson (1975). Somatostatin was measured with radioimmunoassay with the following characteristics. Tyrosylsomatostatin was labelled with 125 I by the lactoperoxidase method and separated on a carboxymethyl-cellulose column. The an

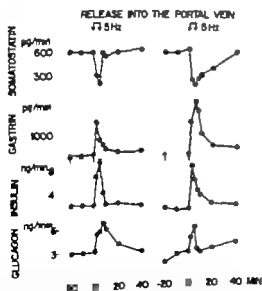


Fig. 1 Release of somatostatin, gastrin, insulin and glucagon into the portal vein following electrical vagal stimulation (5 Hz). Note that somatostatin output decreases whereas the release of gastrin, insulin and glucagon increases (Expts 6 and 5 from Table 1).

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DISCUSSION

The present study demonstrates that electrical stimulation of the vagal nerves was accompanied by a decrease in the release of somatostatin into the portal vein. Simultaneously in agreement with previous findings, vagal stimulation enhanced the release of insulin, glucagon and gastrin into the portal vein (Frohman, Ezdinly & Javid 1967; Kaneto, Kosaka & Nakao 1967; Kaneto, Mikl & Kosaka 1974; Lanceluait et al 1973; Becker, Reeder & Thompson 1974; Smith et al 1975; Uvnäs-Wallensten & Nilsson 1977; Uvnäs-Wallensten 1976; Uvnäs-Wallensten, Uvnäs & Nilsson 1976) and of somatostatin and gastrin into the antral lumen (Uvnäs-Wallensten 1977; Uvnäs-Wallensten, Efendic & Luft 1977). We have previously demonstrated that the intraluminal release of gastrin and of somatostatin from the antrum and duodenum in cats is pH dependent (Uvnäs-Wallensten 1977; Uvnäs-Wallensten, Efendic & Luft 1978). Thus during perfusion of antral or duodenal pouches with neutral or slightly alkaline solutions, electrical stimulation caused a release of gastrin into the lumen, whereas no or minute amounts of somatostatin appeared in the perfusates. During perfusion with 0.1 M HCl, on the other hand, clearly reduced amounts of gastrin and large amounts of somatostatin could be demonstrated in the perfusates. The latter observation was confirmed in the present study. Here, when the pH of the antral perfusate was kept alkaline when vagal stimulations were performed, as expected, only minor amounts of somatostatin but large amounts of gastrin were released (Table 1 and Fig. 2). During perfusion with 0.1 M HCl, on the other hand, substantial quantities of somatostatin appeared in the perfusates.

Electrical vagal stimulation releases gastrin both into the lumen and into the circulation (Uvnäs-Wallensten 1977). It is reasonable to assume that antral somatostatin, in addition to being released into the antral lumen, is also released into the portal vein following vagal stimulation at low intra-antral pH, just as electrical vagal stimulation causes a bidirectional release of gastrin (Fig. 2). Studies performed by Gustavsson & Lundqvist (1978) and Schurdzziarra et al (1978) showing that instillation of acid into the antral lumen enhances the levels of somatostatin in the portal vein, confirm this assumption.

But since portal somatostatin levels in fact decrease in response to electrical vagal stimulation the

lowering of somatostatin levels in the portal vein must be caused by a diminished release of somatostatin from another larger source of somatostatin, thus masking a release of somatostatin from the antrum.

One possibility could be that the release of pancreatic somatostatin is decreased by vagal stimulation. In favour of this idea is the fact that acetylcholine and β -endorphine inhibit somatostatin release from the perfused pancreas of the dog (Sano et al 1977; Ipp, Dobbs & Unger 1976).

Another possible origin of the decreased somatostatin output could be the fundic region of the stomach, since according to Schurdzziarra et al (1978) the output of somatostatin from the antral and fundic regions of the stomach do not always parallel each other.

From a functional point of view this latter idea seems attractive. Vagal stimulation is a potent stimulator of gastric acid secretion and somatostatin is an efficient inhibitor of gastric acid secretion.

A decreased release of the fundic somatostatin might therefore contribute to the stimulatory effect on gastric acid secretion induced by vagal activation. Whether the postulated inhibitory action of somatostatin is exerted locally or via the circulation or by both mechanisms remains to be established.

Since it would be impossible to sample pure pancreatic or fundic blood in cats without severe interference with the rate of blood flow and therefore peptide concentrations, the question as to the antral source of portal vein somatostatin content has to be solved in experiments performed on human animals.

It may be considered justified to attribute the decrease in portal somatostatin levels to an action of vagal cholinergic fibers.

However, recently the vagal nerve has been shown to contain several types of peptidergic fibres including gastrin, somatostatin, VIP, substance P and enkephalins (Uvnäs-Wallensten et al 1977; Uvnäs-Wallensten, Efendic & Luft 1978; Lundberg et al 1978). Therefore it is possible that a vagally induced decrease of portal vein somatostatin level is mediated via peptidergic fibers. This is supported by the finding that in the cat, neither vagally induced release of insulin, glucagon or gastrin into the portal vein, nor the vagally induced release of gastrin and somatostatin into the antral perfusate could be blocked by atropine (Uvnäs-Wallensten 1976; Uvnäs-Wallensten & Andersson 1977).

Effects of phasic and tonic activation on contraction dynamics in smooth muscle

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Responses to isometric quick releases of rabbit urinary bladder strips and rat portal veins activated by AC-stimulation and K⁺-high medium were studied. The AC-stimulation was adjusted to give the same tension as the K⁺-contractures. Releases were performed at peak of the contractions (which was attained after 1.5-4.5 AC-stimulation or 2-3 min in K⁺-high solution). The length response consisted of 3 parts: (1) elastic recoil, (2) isometric transient, (3) steady shortening. Shortening velocity was determined at 100 ms after the release as phase (2) had subsided by then. Characteristics of the initial elastic response was virtually unaffected by the mode of stimulation for both preparations. V_{max} was significantly higher for the AC-stimulations than for the K⁺-contractures (bladder 0.37 l/s vs. 0.26 l/s, portal vein 0.46 l/s vs. 0.33 l/s). By means of computer analysis that exponential shortening component comprising the larger part of phase (2) could be separated from the subsequent slower shortening (see further in Hellstrand & Johansson 1979). In each preparation the amplitude of this exponential was the same for both modes of activation. The time constant was, however, smaller for the AC-stimulated preparations. Our results thus indicate that in response to sudden decrease in force the initial elastic recoil is the same whereas the rate of transition to steady shortening, and the steady shortening velocity itself are lower for preparations activated by K⁺-high medium compared to AC-stimulation.

Key words: Urinary bladder, portal vein, series elasticity, force-velocity relation, mechanical transients, electrical stimulation, K⁺-contraction.

The length response to a step change of force in a muscle consists of an initial elastic recoil, followed by a phase of slower active shortening (e.g. Jewell & Wilkie 1958). In skeletal muscle it has been shown that the second phase of shortening is influenced in its earliest part by phenomena resembling viscoelastic relaxation of complicated form (Podolsky 1960; Crain & Podolsky 1966; Birley 1974). Corresponding phenomena are seen in the force response to a length step and interpretation of these effects are attempted in terms of the cross-bridge mechanism of contraction (Podolsky 1966; Zaveri 1969; Huxley & Simmons 1971; Noble & Thorson 1973; Abbot & Steiger 1977). Recently it has been demonstrated that phenomena of similar appearance are encountered in smooth muscle although their time course is much slower and it cannot yet be regarded as certain that

they correspond to those in skeletal muscle (Johansson, Hellstrand & Uvelius 1978; Hellstrand & Johansson 1979; Mulvany 1979). One consequence of the presence of the viscoelastic properties is that the velocity of shortening in the second phase of the length response is greatly dependent on time after the release. In order to specify the force-velocity relation it is thus necessary to measure shortening velocity at a fixed point in time when the viscoelastic phenomena have essentially subsided (Hellstrand & Johansson 1979).

The immediate series elastic recoil in smooth muscle is several times larger than what is found in skeletal muscle (e.g. Bressler & Clinch 1974; Ford, Huxley & Simmons 1977). It is unlikely that major part of the responses is to be ascribed to recoil of the individual cross-bridges, as is proposed to be the case in skeletal muscle. However, a small por-

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frequency of 1 kHz and analysed by computer program that performed fit of the length record (extrapolated to the elastic recoil, to double exponential function of time).

$$L(t) = A_1[1 - \exp(-t/\tau_1)] + A_2[1 - \exp(-t/\tau_2)] \quad (1)$$

Each exponential component is then characterized by its amplitude (A_i) and its time constant (τ_i). The elastic recoil was computed by back extrapolation of the fitted curve to its first intersection with the record (cf. Fig. 1). The magnitude of the force step was also calculated by the computer. The fit to the length record was performed when chosen time interval beginning about 8 ms after the force step and extending until 500 ms after the step. Occasionally the starting point had to be changed due to irregularities in the record or prominent oscillations, particularly at low afterload and for the portal vein records (where the elastic recoils are greater).

Apart from the elastic recoils and the parameters of the responses the program calculates, by differentiation, the shortening velocity at any desired point in time within the material. In this work all velocities are measured at 100 ms after the release at each time the first exponential component (time constant 20–40 ms) has essentially subsided. The velocities (V) and afterloads (P) were fitted by computer program to the Hill (1936) equation:

$$V = P/(P + a) \quad (2)$$

The relation characterized by the constants a , b and P (except on force axis) is accord with common usage. The dimensionless quantity a/P is given here. The maximal shortening velocity (at zero load) is obtained as $V_{\infty} = b/a$.

The elastic properties of smooth muscle are well described by simple exponential force-extension relation. The release data were fitted to logarithmic form of the relation:

$$\Delta L/L = \ln \frac{P + B}{A} \quad (3)$$

where A , B and k are constants and L is the initial muscle length.

The general procedure of the experiments was as follows. After detension and mounting at preload of <0.2 mN (bladder) or <0.8 mN (portal vein), the muscle was allowed to recover for at least 45 min in tris-buffered Krebs solution of the following composition in mM: NaCl 118, KCl 4.0, MgCl₂ 1.2, CaCl₂ 2.5, glucose 11.5, CaNa₂EDTA 0.026 and tri-(hydroxymethyl) aminomethane (Tris) Base (Sigma Chemical Co.) 23. The solution was treated with HCl to pH of 7.4 at 37°C and was bubbled with O₂. The osmolality was about 290 mosm/kg H₂O. After the equilibration period the bath was exchanged for one containing solution where 100 mM of NaCl had been replaced with KCl. In this K⁺-high solution, contracture develops. Each usually consists of an initial peak, followed by partial relaxation and second slower rising phase, reaching its maximum in about minute (Sapich & Ulfendin 1977). After repeated exposures to K⁺-high and normal solution at rapid time schedule (2–3

min contraction, 5 min relaxation), highly reproducible responses were obtained. Isotonic releases were then performed when the second phase of the isometric responses had reached its maximum. Afterloads were varied in a random fashion. After all releases had been recorded the muscle was kept in normal solution and electrical stimulation (25–100 Hz, 2–4 V, duration 2–5 s) at 1–1 min intervals, was begun. Usually the maximal tension response to AC stimulation was somewhat greater than that in K⁺-high solution. To enable comparisons of shortening velocities at similar degree of activation of the muscle, the stimulation parameters (frequency and amplitude) were adjusted to give AC responses of similar force as the previously obtained K⁺ contractures. The reduction in contractile tension was slight, but it should be emphasized that in this study the muscles were not usually maximally activated during the responses to electrical stimulation. When steady AC responses had been obtained, isotonic releases at peak of contraction were performed in similar sequence as for the K⁺ contractures.

Statistical comparisons between K⁺ and AC responses in the individual muscles were made according to Student's *t*-test for paired values. Amplitudes and time constants of the postload component in the total material were grouped according to P/P into classes with width of 0.2. Comparisons of K⁺ and AC data between corresponding classes were made by Student's *t*-test for unpaired values. Significance levels are indicated as: $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

RESULTS

Fig. 1 shows computer displays of force and length records in a portal vein subjected to similar force steps in a K⁺-contracture (K) and an AC-electrically stimulated contraction (AC). Two different time intervals of the same releases are shown (100 ms and 500 ms). Broken line shows a computer fit according to a double exponential model (see Methods). Delineation of the initial elastic length response is shown by the intersection of the fitted line with the length record. The rest of the shortening response is treated as sum of two exponentials with time constants 37 ms and 0.61 s (k_1) and 29 ms and 0.33 s (AC) respectively. (1) the following the responses are treated in this order: (1) The initial elastic recoil (SE) (2) the shortening velocity at 100 ms after the release (V) and (3) the amplitudes and time constants of the exponential components of shortening.

1. Series elasticity

The initial (series elastic) recoil for different afterloads were determined in the manner described above. Fig. 2 shows the results for one bladder preparation. Open circles show elastic recoil for

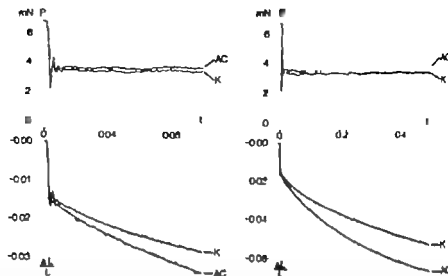


Fig. 1. Display of digitized record and curve fits of isotonic quick releases on a portal vein during AC stimulated contraction (AC) and K^+ -induced contracture (K^+). Left and right panels show same releases on different time scales. In each panel top records shows force and bottom records length change (shortening downward). Broken line shows fit to length record according to double exponential model eq. (1).

tion of the elastic response in urinary bladder smooth muscle seems to be dependent on the state of activation of the contractile system and may be associated with cross-bridge recoil (Hellstrand & Johansson 1979).

It has repeatedly been suggested that shortening velocity in smooth muscle is lower if the muscle is activated by K^+ high medium rather than by electrical stimulation (Hardung & Laszt 1966; Hellstrand & Johansson 1975; Murphy 1976). Whether this is due to special effects of the K^+ high medium or to differences between "tonic" and phasic activity appears not to be known. Detailed comparisons in individual muscles have not been reported. We considered that this phenomenon might contribute to the understanding of the dynamic properties of smooth muscle. In the present study series elasticity, force-velocity relations and isotonic transient responses have been characterized in smooth muscle from rabbit urinary bladder and rat portal vein. Contractions were elicited by K^+ high solution and by alternating current (AC) electrical stimulation. Care was taken to ensure equal isometric tension in the same muscle for both kinds of activation. Isotonic quick releases were performed at peak of the contractions.

The reason for the use of both the rabbit bladder and the rat portal vein is that, in addition to being from different species, they represent different types of smooth muscle. The portal vein is sponta-

neously active whereas the bladder is much more controlled by its rich autonomic nerve supply. Mechanical behaviour common to these preparations can therefore, with greater certainty be considered relevant for mammalian smooth muscle in general. A quantitative study of isotonic transient responses to quick release in bladder muscle was done by Hellstrand & Johansson 1979. Here we report such measurements on the portal vein as well. Some preliminary results have been presented earlier (Uvelius & Hellstrand 1980).

METHODS

Preparations of smooth muscle from rabbit urinary bladder and rat portal vein were dissected as described by Uvelius (1976) and by Hellstrand & Johansson (1979). Each muscle strip had a length of 3–7 mm and was mounted in the apparatus described by Sjölén, Hellström & Clementz (1978) and by Hellstrand & Johansson (1979). This apparatus allows force steps from isometric contractions to a constant afterload to be accomplished in less than 1 ms (cf. Fig. 1). The muscle is mounted horizontally in a thermoregulated trough (1.5 ml) perfused with oxygenated solution (1 ml/min) and equipped with platinum electrodes for electrical stimulation. Three different individually perfused baths can be rapidly exchanged allowing quick change of solution. The temperature of the bath media was kept at 37°C. It was checked that AC stimulation did not alter temperature in the medium surrounding the muscle. Force and length signals were recorded on a storage oscilloscope, on a paper recorder and on magnetic tape for later computer analysis.

The isotonic release records were digitized at a sam-

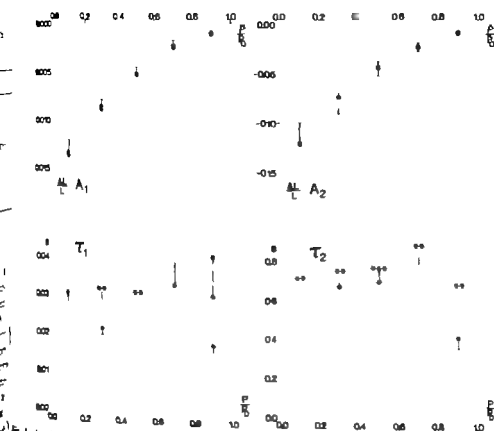


Fig. 4. Amplitudes (A_i) and time constants (τ_i) of the two exponential components ($i=1, 2$) of eq. (1). Bladder strips. Filled circles: AC stimulation, open circles: K⁺ activation. Data grouped into classes according to P/P_0 . Each point represents the mean \pm S.E. for 9-11 results from 6 strips.

of the portal vein, expressed in muscle lengths per second, is somewhat greater than in bladder. The relative difference in V_{max} between K⁺ and AC is about the same in both muscles, 30% lower in K⁺.

Exponential component 1 of shortening

The shortening response after the initial elastic recoil is analyzed according to a model consisting of a combination of two exponential processes, one "fast" and one "slow". The fast component was suggested by Hellstrand & Johansson (1979) to represent a transient phase of cross-bridge rearrangement and change of turnover rate. The slow component was considered to represent the steady-state isometric response. Some theoretical justification for the choice of an exponential function to describe isometric shortening exists, but in the actual experimental effects such as temporal variations in activation, possible inactivation due to shortening (cf. results from skeletal muscle by Edman 1976) and

non-linear length-tension relations complicate the interpretation of the second phase. In the present context, the analytical description of this phase is used for obtaining the shortening velocity at any desired point in time and as a background for deduction of the fast exponential component (Hellstrand & Johansson 1979).

Fig. 4 shows the amplitude (A_i) and time constants (τ_i) of the "fast" ($i=1$) and slow ($i=2$) components obtained from the expts on bladder muscle. A_1 is very similar for the two kinds of activation over the entire range of P/P_0 . On the other hand τ_1 is consistently greater in the K⁺ responses. The same pattern was seen for A_2 and τ_2 although these parameters are about one order of magnitude greater.

The results from portal vein are shown in Fig. 5. A_1 and τ_1 are rather similar to the bladder data although there is more spread and τ_1 varies with P/P_0 in a slightly different way. On the other hand

Table 1 Mechanical parameters of bladder and portal vein during AC and K⁺ activation

P and V_{max} were determined as the intersections of the force-velocity hyperbola with the force and velocity axes respectively. k =stiffness of the elastic element from eq. (3). a/P and b =parameters of Hill's eq. (7). Velocity measured 100 ms after release. Mean values \pm S.E.

| | P (mN/mm ²) | k | V_{max} (U/s) | a/P | b (U/s) | n |
|-------------|-------------------------|------------------|-----------------|-----------------------|-----------------------|-----|
| Bladder | | | | | | |
| AC | 35 \pm 4 ns | 51 \pm 6 ns | 0.37 \pm 0.05 | 0.33 \pm 0.06 ns | 0.11 \pm 0.01 ns | 6 |
| K | 34 \pm 4 | 54 \pm 3 | 0.76 \pm 0.03 | 0.40 \pm 0.09 | 0.10 \pm 0.02 | |
| Portal vein | | | | | | |
| AC | 23 \pm 7 ns | 32 \pm ns | 0.46 \pm 0.04 | 1.4 \pm 0.59 n | 0.46 \pm 0.19 ns | 7 |
| K | 71 \pm 1 | 37 \pm 4 | 0.33 \pm 0.0 | 1.7 \pm 0.19 | 0.53 \pm 0.07 | |

releases performed during K⁺ activation and filled circles those during AC stimulation. Fits of the elastic length responses to exponential curves are shown. The K⁺ recoil seems to be somewhat smaller for identical force steps, but although this was the most common finding it was not consistent. The exponential relation may be characterized by the dimensionless parameter k of eq. (3). This is shown in Table 1. Neither for bladder nor portal vein is there any significant difference in k between K⁺ and AC, although the means for K⁺ are greater in both muscles. Bladder muscles are generally stiffer than portal veins.

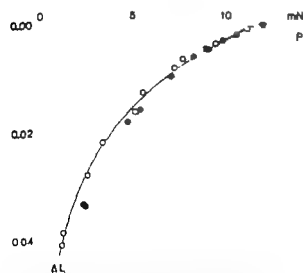


Fig. 2 Series elastic recoils of a bladder strip in releases from an isometric tension of 12 mN to different afterloads P. Filled circles: AC stimulation; open circles: K⁺ activation. Exponential fits to both sets of data are shown by broken and full line, respectively. Constant k of eq. (3) is 43 for AC and 50 for K⁺.

AL: P (mN) 4.5 and 10.9

Force-velocity relations

Fig. 3 shows shortening velocity at 100 ms after release (see Methods) plotted against the afterload P for a bladder strip where the mean isometric tension before release was 11.93 mN in K⁺ and 12.1 mN in AC. The curves fitted according to Hill's eq. (7) intersect with the force axis at points somewhat above these values. This was seen in most experiments. The P values in Table 1 are those obtained from the intersection of the hyperbola with the force axis. The shortening velocities in AC are consistently greater than in K⁺ for similar afterloads. The V_{max} values in the two kinds of contraction are significantly different both for bladder and portal vein (see Table 1). For the same kind of stimulation the V_{max}

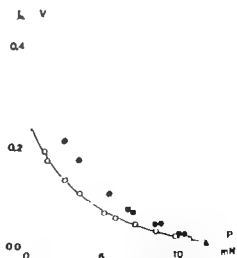


Fig. 3 Force-velocity relations of bladder strip. Velocities measured 100 ms after release. Fits of Hill's eq. (7) shown. Filled circles: broken line: AC stimulation; open circles: full line: K⁺ activation.

Li & Smith (1973) have reported that cells in smooth muscle, such as rabbit portal-mesenteric vein, will in solutions where KCl has been substituted for NaCl. They also observed disruptions of actin filaments in muscle fixed after KCl, but argued that this occurred during the fixation as the contractile response in KCl and K_2SO_4 (which did not cause disruption) were the same. It is possible that cell swelling and filament disruption (if such occurs also in the unfixed muscle in KCl) might be of relevance in explaining the V_{max} differences. There even now over a difference in time course between the K- and the AC-constrictions as used in the study. While the latter are of short duration (~ 1 s) the K-constrictions last for ~ 3 min before the release is made. It is possible that V for a given load increases with time irrespective of the mode of stimulation. Such an effect has been described in *Limacris* (1978) on slow muscle fibres of *Limacris*. Moreover it has been shown for portal vein by Hellstrand & Johansson (1975) and for urinary bladder by Ulfhake (1979) that V_{max} during a single twitch decreases more rapidly with time than does the extrapolated P_0 . It is known that in portal vein (Hellstrand 1977) the metabolic cost for producing a given isometric tension is higher for phasic than for K-constrictions. Furthermore in K-constrictions O_2 consumption in relation to tension development decreases with time (Arner & Ekblom 1980). It appears possible that an insufficient ATP supply will occur in muscles contracted for longer periods. This might lead to an increase in the life time of attached cross-bridges, possibly by formation of rigor complexes (Weber & Murray 1977).

For both muscles the amplitude (A) of the fast shortening component is unaffected by the mode of stimulation. Similarly Hellstrand & Johansson (1975) found that A was unaffected by level of activation and by temperature. The fast shortening component is interpreted to represent conformational changes in attached cross-bridges, and possibly transient changes in the balance between attachment and detachment of cross-bridges occurring after the force step. It is reasonable to expect factors which affect the kinetics of cross-bridge turnover to influence primarily the time course of the force, whereas its amplitude will be determined by the size of the force step.

The time constant ($1/\tau$) of the fast shortening component is for both preparations about 55%

smaller in K-constrictions. As mentioned above V_{max} is about 30% lower in K-constrictions. Thus the K-constriction affects the rate constant ($1/\tau$) and V_{max} in a comparable way as cooling of the muscle. The similarity in relative difference between $1/\tau$ and V_{max} for the two modes of activation in this study provides further evidence that the fast shortening component is not a passive phenomenon but has relation to the contractile system.

If the level of activation could be held constant long enough after the release, the amplitude (A) of the slow exponential component would indicate the amount of shortening needed to reach points on the length-tension curve corresponding to the different afterloads. The time constant (τ) of the component would be determined solely by the force-velocity relation, the length-tension relation, and the afterload. In the actual experiment, effects such as decrease with time in the level of activation complicate the interpretation. τ is larger for K-constrictions in both preparations, which is to be expected from the change in the force-velocity relation. In bladder A_2 is the same for both stimulations, whereas in portal vein it is larger for AC-constrictions. It seems possible that in portal vein the passive tension which is not negligible might influence the length-tension relation in such a way that the exponential approximation is inapplicable, although within a restricted time interval the fits are excellent.

The present study has shown that a homogeneously activated smooth muscle can have V_{max} values that differ with the mode of activation despite similar P_0 and elastic properties. The transient isotonic component of shortening has a slower time course in K-constrictions, in accordance with the suggestion that it represents relaxation phenomena within the cross-bridge system in response to the force step. The reason for the slower kinetics in K-constrictions is not revealed by the present study. Besides effects of the K-high solution as such, the longer duration of the contraction before release may be of importance.

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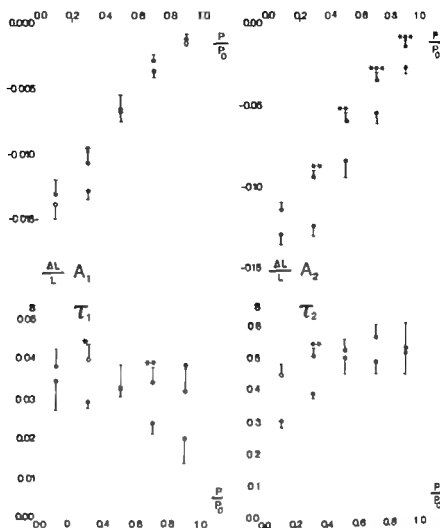


Fig. 5. Amplitude (A) and time constant (τ) of the two exponential components ($i=1, 2$) of eq. (1). Portal veins: filled circles; AC stimulation: open circles. Data grouped according to P/P_0 . Each point shows mean \pm S.E. $n=9-13$. Result from 7 expts.

there is a consistent difference in A_2 between K and AC expts. the amplitudes being numerically greater for the latter. τ_2 is greater for K , as in urinary bladder.

Although the amplitude of the fast exponential component is the same for both modes of stimulation, its time course is different, as seen by the fact that the rate constant ($1/\tau$) was on the average 25% smaller during K -contracture, both in bladders and portal veins.

DISCUSSION

The series elastic element (SE) is stiffer in urinary bladder smooth muscle than in portal vein. This has previously been shown by Johansson, Hellstrand & Uvelius (1978). As is shown in Table 1 there is no significant difference in SE stiffness of either mus-

cle for the two kinds of stimulation, although there is a tendency towards a higher k for the K -contractures in both preparations. The major portion of the SE undoubtedly resides in passive structures. However, the results of Hellstrand & Johansson (1979) suggest that part of the recoil (1% or less of the muscle length) occurs in structures associated with the crossbridges. If a difference in this fraction of the SE exists between the two kinds of activation, it will probably be masked by the large recoil of passive structures, and the fact that some variation in P between individual contractions occurred.

For the same kind of stimulation, the portal veins shorten faster than the bladders. For both preparations V_{max} for K -contractures are about 30% lower than for AC stimulations (Table 1), in spite of almost the same tension development. Jones & Som-

Amylase secretion in response to activation of different autonomic receptors in the rabbit parotid gland

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The contribution by different autonomic receptors to the amylase secretion from the parotid gland of the anesthetized rabbit was studied as the response to various parasympathetic and sympathetic drugs. Amylase secretion by infusions of pilocarpine or parasympathetic nerve stimulation was low but regularly higher in response to pilocarpine than to parasympathetic nerve stimulation. These effects were reduced to the same level by β -adrenoceptor block indicating the presence and for pilocarpine also the release of catecholamines probably from the adrenal. Isoprenaline injections produced high amylase secretion, that was blocked by atropine indicating that predominantly α -adrenoceptors were activated. Phenylephrine was without amylase secretory effects. By accepting isoprenaline estimates as maximum for sympathetic produced amylase secretion, theoretical frequency-response relationship for amylase secretion by sympathetic nerve stimulation could be calculated. $ED_{50} = 0.9$ Hz. The results indicate that under experimental conditions *in vivo* there are certain differences between the rat and the rabbit parotid glands in the autonomic control not only of fluid but particularly of amylase secretion.

Key word: Rabbit, parotid gland, amylase secretion, α - and β -adrenoceptors, muscarinic receptor.

Amylase secretion in salivary glands has mainly been studied in the rat parotid gland. *In vitro* amylase is predominantly released on β -adrenoceptor activation, but some amylase is also obtained on stimulation of α -adrenoceptors or muscarinic receptors (see Schönborn & Selinger 1975). However in *in vivo* the situation is more complex in that the output of amylase by parasympathetic nerve stimulation can be as great as that by sympathetic activation (Garrett & Thulin 1975; Garrett, Thulin & Kidd 1971). Fluid secretion like in most salivary glands (see Schneyer & Emmelin 1974) is predominantly evoked by parasympathetic nerves, but a slow sustained flow of saliva can be obtained also on sympathetic nerve stimulation, an effect controlled via both α - and β -adrenoceptors (Garrett & Thulin 1975; Thulin 1975). About the rabbit parotid gland less is known, but *in vivo* experiments indicate that parasympathetic nerve stimulation evokes secretion of fluid with content of amylase. The amylase secretion can mainly be under the control of the sym-

pathetic nerves, and in the presence of a parasympathetic salivary flow large amounts of amylase can be released at stimulation frequencies too low to evoke any fluid secretion. The sympathetic fluid secretion is sparse and mainly mediated via α -adrenoceptors (Gjörstrup 1977, 1978, 1979).

The present experiments set out to more closely investigate to what extent activation of different autonomic receptors contributes to the secretion of amylase from the rabbit parotid gland. The effects of injections of various autonomic drugs were studied rather than sympathetic nerve stimulation. In this way it was possible to overcome vasoconstriction which particularly in the rabbit salivary glands, tends to severely retard the fluid secretion necessary to transport the amylase (see Gjörstrup 1979).

METHODS

30 rabbits, 1.7-3.4 kg and kept on standard pelleted diet, were used. 22-26 h prior to the experiments the animal

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Amylase secretion in response to activation of different autonomic receptors in the rabbit parotid gland

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The contribution by different autonomic receptors to the amylase secretion from the parotid gland of the anaesthetized rabbit was studied as the response to various parasympathomimetic and sympathomimetic drugs. Amylase secretion by infusions of pilocarpine or parasympathetic nerve stimulation was low but regularly higher in response to pilocarpine than to parasympathetic nerve stimulation. These effects were reduced to the same level by β -adrenoceptor block indicating the presence and for pilocarpine also the release of catecholamines, probably from the adrenals. Isoprenaline injections produced high amylase secretion, that was blocked by atenolol, indicating that predominantly β_1 -adrenoceptors were activated. Phenylephrine was without amylase secretory effects. By accepting isoprenaline maximum as maximum for sympathetically produced amylase secretion, theoretical frequency-response relationship for amylase secretion by sympathetic nerve stimulation could be calculated. ED_{50} was 0.9 Hz. The results indicate that under experimental conditions *in vivo* there are certain differences between the rat and the rabbit parotid glands in the autonomic control not only of fluid, but particularly of amylase secretion.

Key words: Rabbit, parotid gland, amylase secretion, α - and β -adrenoceptors, muscarinic receptors

Amylase secretion in salivary glands has mainly been studied in the rat parotid gland. *In vitro* amylase is predominantly released on β -adrenoceptor activation, but some amylase is also obtained on stimulation of α -adrenoceptors or muscarinic receptors (see Schramm & Selinger 1975). However, in *in vivo* the situation is more complex in that the output of amylase by parasympathetic nerve stimulation can be as great as that by sympathetic activation (Garrett & Thulin 1975; Garrett, Thulin & Kidd 1976). Fluid secretion, like in most salivary glands (see Schneyer & Emmelin 1974), is predominantly evoked by parasympathetic nerves but a slow sustained flow of saliva can be obtained also on sympathetic nerve stimulation, an effect conveyed via both α - and β -adrenoceptors (Garrett & Thulin 1975; Thulin 1975).

About the rabbit parotid gland less is known, but no experiments indicate that parasympathetic nerve stimulation evokes secretion of fluid with content of amylase. The amylase secretion seems mainly to be under the control of the sym-

pathetic nerves and in the presence of a parasympathetic salivary flow large amounts of amylase can be released at stimulation frequencies too low to evoke any fluid secretion. The sympathetic fluid secretion is sparse and mainly mediated via α -adrenoceptors (Gjörstrup 1977, 1978, 1979).

The present experiments set out to more closely investigate to what extent activation of different autonomic receptors contributes to the secretion of amylase from the rabbit parotid gland. The effects of injections of various autonomic drugs were studied, rather than sympathetic nerve stimulation. In this way it was possible to overcome vasoconstriction, which, particularly in the rabbit salivary glands, tends to severely retard the fluid secretion necessary to transport the amylase (see Gjörstrup 1979).

METHODS

30 rabbits, 1.2-3.4 kg and kept on standard pelleted diet, were used. 22-26 h prior to the experiments the animal

were deprived of food but allowed water ad libitum. In the acute experiments anaesthesia was induced by injecting urethane 1.5–1.7 g/kg into a lateral ear vein; additional doses were given through a cannulated femoral vein. Elimination of the corneal reflex was considered a sign of a satisfactory depth of anaesthesia. A tracheal cannula was inserted. In 5 rabbits the right superior cervical ganglion was removed in pentobarbitone anaesthesia 35 mg/kg i.v. 3 to 4 days prior to the acute experiment. This time allowed for the gland to be reloaded with amylase after the sympathetic degeneration secretion that follows ganglionectomy (Garrett & Thulin 1975b).

To register the rate of fluid secretion and to collect saliva for amylase determination the right parotid duct was cannulated in close to its orifice in the mouth as possible. Glass cannulae giving drops of a size of 15 μ l for distilled water were used. The drops were recorded on a smoked drum by an ordinate writer manually operated. Parasympathetic fluid secretion was evoked by electrical stimulation (supramaximal intensity, ms duration) of the auriculo-temporal nerve which was dissected as described by Burgen (1964) and Navrocki (1968). Drugs were injected or infused either i.v. through the femoral vein or i.a. via the lingual artery after ligation of all major branches from the external carotid artery including the facial artery distal to the parotid gland. The blood flow through the submaxillary gland was eliminated by removing the gland. The i.a. route was regularly used for α -blocking drugs to avoid the fall in blood pressure that is particularly marked in the rabbit in i.v. injections (Harvey & Nickerson 1953; Cjörström unpublished). In 9 acute experiments adrenalectomy was performed via a mid line abdominal incision. The aortic supply was either ligated and the adrenals were removed or the vessel were clamped leaving the adrenals in situ depending on the access to the gland.

Drugs were injected either i.a. into a non-secreting gland or in the presence of a parasympathetic fluid secretion imitating the resting secretion normally present in the waking animal (Schaefer & Emmelin 1974). As has been shown previously 50–100 μ l/min is a suitable background flow of saliva and was evoked by parasympathetic stimulation at 1–2 Hz (Cjörström 1979). Saliva for amylase determinations was always collected over 5 min. When drugs were injected while parasympathetic secretion was going on the experimental schedule was as follows. For each dose of a drug to be used a control sample of parasympathetic saliva was first obtained. At the precise end of that period the drug was injected and saliva for a second period of 5 min was collected. To make allowance for dead space in the cannulae and the time for the drug to reach the gland the first drops formed during this period were regularly omitted. To make sure that the effect of blocking the injection of the stimulating drug had worn off another 5 min passed before the procedure was repeated.

The samples of saliva were stored in deep freeze until assayed according to the method described by Dahlqvist (1964). In this method 1 unit of amylase is equivalent to the amount that liberates reducing groups corresponding to 1 ml fromole of maltose formed each minute at 55°C. Samples of saliva of 50 or 100 μ l were used and diluted 100–1000 times before processing.

Amylase obtained in the saliva is expressed as sample concentration of amylase (units/ml) and as output of amylase per min (units/min).

Statistics

Student's *t*-test for paired and unpaired observations was used to test significance. *P* values less than 0.05 were considered significant.

Drugs

Pilocarpine hydrochloride, adrenaline bitartrate, noradrenaline bitartrate, phenylephrine hydrochloride, heparin sodium sulphate, salbutamol (obtained from Allen &; Overy), atenolol (ICI), dihydroergotamine (Sandoz).

RESULTS

Parasympathetic activation

The parasympathetic saliva that served as a background flow for injections of various sympathetic and metabolic drugs showed an amylase concentration of 433 ± 38 units/ml (mean \pm S.E., $n=6$). A continuous background flow of saliva of 50–100 μ l/min could also be obtained on pilocarpine infusions at 2–3 μ g/kg/min i.v. however the amylase concentration in this saliva was higher being 644 ± 60 units/ml (<0.01 , $n=6$). Extirpation of the superior cervical ganglion 3–4 days prior to pilocarpine infusion further increased the amylase concentration in the saliva 1001 ± 51 units/ml (<0.001 , $n=5$). When pilocarpine 10 μ g/kg was injected i.a. and salivary collected at flow rates of 80–100 μ l/min the amylase concentration was of the same order as that in response to parasympathetic activation being 351 ± 25 units/ml ($n=3$). After β_1 -adrenoceptor block by atenolol 2 mg/kg the amylase concentration in response to parasympathetic nerve stimulation or pilocarpine administration fell to values between 22 and 19 units/ml. Nine attempts were made to study amylase secretion evoked by auriculo-temporal nerve stimulation or pilocarpine infusion after adrenalectomy. However only one experiment was successful despite accurate and fast surgical procedures; the rabbits died in vascular shock within 15–30 min after elimination of the second adrenal. In the successful experiment the amylase concentration produced by auriculo-temporal nerve stimulation was 35 units/ml (mean of 9 observations) before and 140 units/ml (mean of 4 observations) after adrenalectomy. The amylase concentration was not further lowered by atenolol 2 mg/kg.

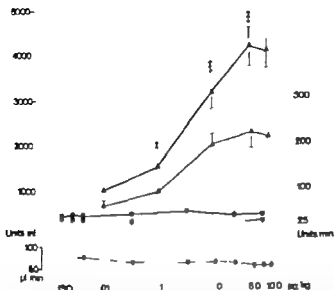


Fig. 1 Amylase secretion in response to various doses of isoprenaline, $\mu\text{g/kg}$. Injections are given against background of parasympathetically evoked fluid secretion that is continuous throughout the experiment. The average secretory rates when samples of saliva were collected is shown (\bullet — \bullet). Each sample of saliva collected after an injection of isoprenaline was preceded by the collection of parasympathetic control sample. Each experiment was begun by collecting 3 control samples. The amylase values are shown separately for those obtained after isoprenaline injections (Δ) and for those which are parasympathetic controls (\square). The amylase secretion is expressed as concentration in units/ml (\rightarrow) and as output in units/min (\dashrightarrow). Mean \pm S.E. from 6 expts. $P < 0.05$ $P < 0.02$; $^{**} P < 0.001$ vs. preceding parasympathetic controls.

Parasympathetic drugs

Isoprenaline, 10–100 $\mu\text{g/kg}$ did not cause any flow of saliva. However the amylase content in the saliva markedly increased when isoprenaline was injected. Parasympathetic secretion 50–100 $\mu\text{g/kg}$, as going on. Already doses as low as 0.1 $\mu\text{g/kg}$ are regularly effective and maximal amylase secretion was evoked by doses around 3 $\mu\text{g/kg}$ (Fig. 1). Superimposed isoprenaline injections did not influence the rate of fluid secretion (Table 1). 2–4 $\mu\text{g/kg}$ markedly lowered or abolished the secretory response to isoprenaline (Fig. 2). Salbutamol, at high doses only 50–100 $\mu\text{g/kg}$, evoked after atropine and with parasympathetic secretion present, caused a minor increase in the amylase concentration in the saliva (Fig. 2) the flow rate was unaffected and on its own salbutamol had no fluid secretory effect at 100 $\mu\text{g/kg}$. Phenylephrine, 30–40 $\mu\text{g/kg}$, administered under the same experimental conditions as those for salbutamol had no effect on amylase or fluid secretion (Fig. 2).

Injections of noradrenaline and adrenaline in the presence of a parasympathetic background secretion caused a dose dependent increase in amylase concentration in the saliva for doses between 0.1–10 $\mu\text{g/kg}$, without changing the rate of fluid secretion (Fig. 3). On high doses 10–30 $\mu\text{g/kg}$, a further increase in amylase concentration was paralleled by decrease in flow of saliva, which in fact decreased the output of amylase per min. The reduction in salivary flow rate to doses of noradrenaline and adrenaline above 10 $\mu\text{g/kg}$ persisted almost unaffected after i.a. injections of dihydroergotamine 0.1–1 mg/kg. On their own noradrenaline and adrenaline were without fluid secretory effects.

Sympathetic frequency-response curve

The intense vasoconstriction seen at sympathetic stimulation above 1 Hz has not made it possible to experimentally obtain frequency-amylase response curve for the whole range of sympathetic stimulation frequencies, so far only the relationship between 0.05 and 1 Hz is known (Gjörstrup 1979).

were deprived of food but allowed water *ad libitum*. In the acute experiments anaesthesia was induced by injecting urethane 1.5–1.7 g/kg into a lateral ear vein; additional doses were given through a cannulated femoral vein. Elimination of the corneal reflex was considered a sign of a satisfactory depth of anaesthesia. A tracheal cannula was inserted. In 5 rabbits the right superior cervical ganglion was removed in pentobarbitone anaesthesia 35 mg/kg *i.v.* 3 to 4 days prior to the acute experiment. This time allowed for the gland to be reloaded with amylase after the sympathetic degeneration secretion that follows ganglionectomy (Garrett & Thulin 1975b).

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RESULTS

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isomol. like phenylephrine was without effect. The results indicate that on sympathetic nerve stimulation amylase secretion is almost exclusively an effect of β -adrenoceptor activation. The amylase secretion remaining in response to low doses of isoprenaline after β -adrenoceptor block, could equally well have been due to an incomplete block by atenolol, as to activation of a sparse number of β -adrenoceptors, indicated by the high doses of isoprenaline needed to obtain some amylase. Similarly to the rabbit, the β -adrenoceptors that mediate amylase secretion in the rat parotid gland are mainly of the β_2 -type but amylase can also be released by drugs known to act more selectively on the β_1 -adrenoceptors (Carlsson, Danielsson & Henselsson 1976). Previous work on the rabbit parotid gland has indicated activation of β -adrenoceptors as the most efficient adrenergic stimulus of amylase secretion (Yamamoto et al. 1968) however variable flow-rates and a different experimental technique make it difficult to judge amylase output and to compare it to the present findings.

Noradrenaline and adrenaline closely resembled sympathetic nerve stimulation in that doses higher than those causing 50–70% of the maximal amylase secretion were followed by a reduced fluid secretion, interpreted as an effect of vasoconstriction. It is not possible to further increase the doses of adrenaline or noradrenaline after α -adrenoceptor block, almost diminishing the fluid secretion to the same extent as seen prior to block indicating, like earlier investigations have done, that sympathetically evoked vasoconstriction is difficult to abolish in the rabbit (Harvey & Nickerson 1953; Gjörrup unpublished).

Isoprenaline on the other hand could be expected to be sufficient to obtain a much larger output of amylase than did adrenaline and noradrenaline. The maximum output for sympathetic nerve stimulation is likely to be of the same magnitude as that in response to isoprenaline since α -adrenoceptor activation has without effect on amylase secretion. The assumption made it possible to calculate a full, hitherto missing, frequency-amylase response curve for sympathetic nerve stimulation. It is reasonable to assume that the theoretical curve could well act as a substitute for an experimentally obtained relationship, since it resembles that seen for autonomic nerves in other glands and organs of different species, having about 90% of the maximum response below 10 Hz and showing

marked response already at 1 Hz (Rosenblueth 1950; Folkow 1952; Folkow, Löfving & Mellander 1956; Carlsten, Folkow & Hamberger 1957; Dammant Enfors & Holmstedt 1959; Emmelin & Holmberg 1967). The difference in slope between the dose-response curve for isoprenaline and the corresponding curve for noradrenaline at sympathetic nerve stimulation may largely be due to the difference in route of drug administration. Electron microscopy of the rabbit parotid gland has shown the sympathetic nerve terminals to be in close proximity with the acini (Bloom, Carlsson & Gustavsson 1976; Garrett 1977). Because the released noradrenaline has a short distance to diffuse it can be assumed to reach the receptors in a high concentration but due to the rapid elimination of the transmitter, high concentration at the receptors is likely to be intermittent on continuous nerve stimulation at low frequencies. Isoprenaline is a relatively long acting substance (Emmelin & Gjörrup 1976) and exogenously administered in single injections its concentration at the receptors is likely to be more even than that of the transmitter and to be dependent on the size of the dose given. Such a difference in drug access and thus in drug-receptor occupancy has been the explanation for a similar difference in frequency response and drug-response curves for noradrenaline mediated contraction of the rat portal vein (Johansson et al. 1971).

Pilocarpine has been reported to cause exocytosis in the rabbit parotid gland (Langley 1879) and in the present experiment it produced amylase secretion. This effect was apparently only to a small extent mediated via muscarinic receptors and mainly via β -adrenoceptors, since most of it disappeared after β -block. In the rat a sympathetic component in pilocarpine evoked parotid saliva was eliminated by acute extirpation of the superior cervical ganglion (Schneyer & Hall 1965, 1966); in the rabbit this procedure three days prior to the experiments increased the amylase concentration. Although only one adrenalectomy was completed the resultant decrease in amylase concentration is suggestive of the adrenals as the source of catecholamines that tend to increase amylase content in parasympathetically or pilocarpine evoked saliva.

The fluid secretory responses when phenylephrine, adrenaline or isoprenaline were injected to the quiescent rabbit parotid gland are in accordance with earlier observations (Gjörrup 1977).

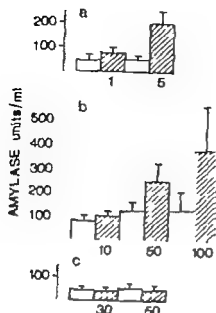


Fig. 2 Effects of isoprenaline (a), salbutamol (b), phenylephrine (c) on amylase secretion doses in µg/kg. The drugs were injected after β -adrenoceptor block by atenolol 2 mg/kg for salbutamol and phenylephrine and 3 mg/kg for isoprenaline. The experiments were performed in the presence of a parasympathetic background flow of saliva 50–85 µl/min. The observation on isoprenaline are from the same experiments as those presented in Fig. 1. Open columns: amylase concentration in preceding parasympathetic controls. All values are mean \pm S.E. of 5–7 expts.

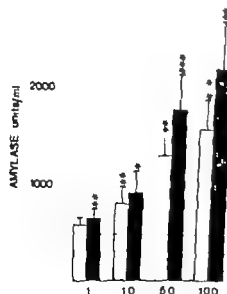


Fig. 3 Amylase secretion in response to noradrenaline and adrenaline µg/kg injected in the presence of a parasympathetically evoked fluid secretion 70–90 µl/min. The scheme shown in Fig. 1 has been followed. Open columns: noradrenaline; black columns: adrenaline. $P < 0.01$, $P < 0.001$ vs. preceding parasympathetic controls. Mean \pm S.E. of 5 expts. Dotted line shows mean amylase concentration in parasympathetic control samples.

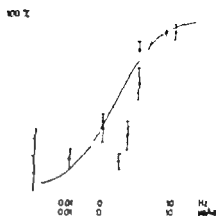


Fig. 4 Frequency-response curve for sympathetic nerve stimulation (broken line) and dose response curve for isoprenaline (continuous line). Amylase secretion in the output units/min in response to individual sympathetic nerve stimulations (from Gjörstrup 1979) and to individual isoprenaline injections (mean values shown in Fig. 1) have been expressed as percentage of mean maximal amylase output by isoprenaline 5 µg/kg (Fig. 1). The percentages have been fitted to a logistic function, for further details see the text. Mean \pm S.E. of 6 expts. with isoprenaline and 7 with sympathetic nerve stimulation.

The previously obtained amylase output/min response to sympathetic nerve stimulation is recalculated as net values by subtracting the amylase output in preceding parasympathetic controls and expressed as percentage of the previously obtained mean isoprenaline maximum (Fig. 1). The amylase secretion evoked by the different isoprenaline doses (Fig. 1) was recalculated in the same way. All values were fitted to a logistic function as devised by Parker & Waud (1971) and resultant theoretical frequency response curve for sympathetic nerve stimulation and its experimental analogue for isoprenaline are shown in Fig. 4. ED_{50} for sympathetic nerve stimulation was 0.9 Hz and for isoprenaline injections 0.18 µg/kg.

DISCUSSION

Noradrenaline, adrenaline and isoprenaline injected in the presence of a parasympathetic fluid secretor could all imitate the amylase secretion seen at sympathetic nerve stimulation under similar conditions (Gjörstrup 1979). The effects of isoprenaline were lowered or abolished by atenolol and after β -adrenoceptor block only a very sparse increase in amylase secretion was seen on injections of sal

laxol, while phenylephrine was without effect. The results indicate that on sympathetic nerve stimulation amylase secretion is almost exclusively an effect of β_2 -adrenoceptor activation. The amylase secretion remaining in response to low doses of isoprenaline after β_1 -adrenoceptor block could equally well have been due to an incomplete block by alaxol, as to activation of a spare number of β_2 -adrenoceptors, indicated by the high doses of alaxol needed to obtain some amylase. Similarly to the rabbit, the β_2 -adrenoceptors that mediate amylase secretion in the rat parotid gland are mainly of the β_2 -type, but amylase can also be released by drugs known to act more selectively on the β_1 -adrenoceptors (Carlsson, Danielsson & Henschen 1978). Previous work on the rabbit parotid gland has indicated activation of β -adrenoceptors as the most efficient adrenergic stimulus of amylase secretion (Yamamoto et al. 1968), however variable dose-rates and a different experimental technique make it difficult to judge amylase output and to compare it to the present findings.

Noradrenaline and adrenaline closely resembled sympathetic nerve stimulation in that doses higher than those causing 50–70% of the maximal amylase release were followed by a reduced fluid secretion, interpreted as an effect of vasoconstriction. It is not possible to further increase the doses of adrenaline or noradrenaline after α -adrenoceptor block, almost diminishing the fluid secretion to the same extent as seen prior to block indicating, like other investigations have done, that sympathetically evoked vasoconstriction is difficult to abolish in the rabbit (Harvey & Nickerson 1959; Gjönrup unpublished).

Isoprenaline, on the other hand, could be injected in doses sufficient to obtain a much larger output of amylase than did adrenaline and noradrenaline. The maximal output for sympathetic nerve stimulation is likely to be of the same magnitude as that in response to isoprenaline since α -adrenoceptor activation was without effect on amylase secretion. The assumption made it possible to calculate a full, hyperbolic, frequency-amylase response curve for sympathetic nerve stimulation. It is reasonable to assume that the theoretical curve could well act as a substitute for an experimentally obtained relationship, since it resembles that seen for autonomic nerves in other glands and organs of several species, having about 90% of the maximum response at 10 Hz and showing a

marked response already at 1 Hz (Rosenbluth 1950; Folkow 1952; Folkow, Löfving & Mellander 1956; Carlsten, Folkow & Hamberger 1957; Diamant, Enfors & Holmstedt 1959; Emmelin & Holmberg 1967). The difference in slope between the dose response curve for isoprenaline and the corresponding curve for noradrenaline at sympathetic nerve stimulation may largely be due to the difference in route of drug administration. Electron microscopy of the rabbit parotid gland has shown the sympathetic nerve terminals to be in close proximity with the acini (Bloom, Carlsson & Gustavsson 1976; Garrett 1977). Because the released noradrenaline has a short distance to diffuse it can be assumed to reach the receptors in a high concentration, but due to the rapid elimination of the transmitter a high concentration at the receptors is likely to be intermittent on continuous nerve stimulation at low frequencies. Isoprenaline is a relatively long acting substance (Emmelin & Gjönrup 1976) and exogenously administered in single injections its concentration at the receptors is likely to be more even than that of the transmitter and to be dependent on the size of the dose given. Such a difference in drug access and thus in drug-receptor occupancy has been the explanation for a similar difference in frequency-response and drug-response curves for noradrenaline mediated contraction of the rat portal vein (Johansson et al. 1971).

Pilocarpine has been reported to cause exocytosis in the rabbit parotid gland (Langley 1879) and in the present experiment it produced amylase secretion. This effect was apparently only to a small extent mediated via muscarinic receptors and mainly via β -adrenoceptors, since most of it disappeared after β -block. In the rat a sympathetic component in pilocarpine evoked parotid saliva was eliminated by acute extirpation of the superior cervical ganglion (Schneyer & Hall 1965, 1966); in the rabbit this procedure three days prior to the experiments increased the amylase concentration. Although only one adrenalectomy was completed the resultant decrease in amylase concentration is suggestive of the adrenal as the source of catecholamines that tend to increase amylase content in parasympathetically or pilocarpine evoked saliva.

The fluid secretory responses when phenylephrine, adrenaline or isoprenaline were injected to the quiescent rabbit parotid gland are in accordance with earlier observations (Gjönrup 1977

Nordenfelt & Ohlin 1957 Ohlin 1964) which have shown only very sparse if any fluid secretory effects on activation of α or β -adrenoceptors by the doses presently used. The same doses of these drugs regularly evoke fluid secretion in the rat parotid gland (Ekström 1973 Ohlin 1964).

The results show that there are certain differences between rats and rabbits in the nervous control of secretion of amylase and fluid from the parotid gland in vivo. In the rabbit amylase secretion is practically only an effect of β -adrenoceptor activation whereas in the rat the output can be as great at parasympathetic nerve stimulation as at sympathetic. Another major difference is that maximal amylase secretion for sympathetic stimuli can be evoked without any accompanying fluid secretion which is the case in the rat. Thus the nervous control of the amylase and fluid secretion in the rabbit can experimentally be more separated than in the rat which may prove helpful for studies on the interactions between parasympathetic and sympathetic nerves in their control of salivary amylase and fluid secretion.

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Effects of carbachol on isoprenaline evoked amylase release from the rabbit parotid gland in vitro

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Amylase secretion from dispersed lobules of the parotid gland of the rabbit was studied in response to isoprenaline (10^{-8} – 10^{-4} M) and to carbachol (10^{-6} – 10^{-4} M). The effects of each agent were investigated alone and in combination at certain concentrations. Isoprenaline produced dose-related amylase secretion with an average maximum of 688 units/100 mg. at 10^{-4} M. The amylase secretion produced by submaximal concentrations of isoprenaline could be further increased by carbachol, already in low concentrations (10^{-6} – 10^{-4} M), that were subthreshold for amylase secretion. This potentiating effect was seen not only as larger secretion but also as greater depletion of amylase from the tissue. In contrast to what is known from experiments *in vivo* carbachol in high concentration (10^{-4} M), by activating muscarinic receptors only released amylase to the same extent as that released by isoprenaline. This concentration of carbachol regularly decreased the amylase secretion evoked by isoprenaline and may be regarded as physiologically high. The increase in isoprenaline evoked amylase secretion brought about by carbachol in lower concentrations, may be due to an improved transport of amylase, because of secretion of fluid, but could also be caused by augmentation of the β -adrenoceptor mediated effects.

Key words. Rabbit, parotid gland, amylase secretion, β -adrenoceptor activation, carbachol, isoprenaline.

Experiments *in vitro* and *in vivo* have established that the release of amylase from parotid salivary glands is predominantly due to activation of β -adrenoceptors (see Schramm & Selinger 1976, Askling et al. 1979, Gjöstrup 1979a, Askling & Gjöstrup 1980). In experiments on the rabbit parotid gland sympathetic nerve stimulation or injections of isoprenaline are found to greatly increase the content of amylase in a parasympathetically produced saliva, already at frequencies or doses below threshold for fluid secretion (Askling & Gjöstrup 1980, Gjöstrup 1977, Gjöstrup 1979). This importance of the parasympathetic nerves for the production of a salivary flow that can transport sympathetically released amylase along the glandular duct has recently been shown also in the conscious animal during feeding (Gjöstrup 1979b). However, it is conceivable that the parasympathetic activity apart from producing fluid may have augmented the β -adrenoceptor mediated amylase secretion, and parasympathetically evoked fluid secretion is known to

be augmented by parasympathetic activity (see Emmelin 1979) and thus has been demonstrated for the rabbit parotid gland also (Gjöstrup 1977).

In an attempt to obtain further knowledge about the promoting action of parasympathetic activity on the sympathetically evoked secretion of amylase the interaction between sympathetic and parasympathetic secretory stimuli was studied in a system that minimizes the intraglandular transport distance for released amylase and thus the dependence of the salivary flow through the glandular duct system, that is necessary *in vivo*. The present experiments describe isoprenaline evoked amylase secretion from dispersed rabbit parotid lobules and how this secretion is influenced by simultaneous carbachol stimulation.

METHODS

Parotid glands from 28 rabbits, 1.5–4.5 kg, were used. The animals were kept on standard pelleted diet and starved but allowed water 24 h prior to the experiments. To remove the

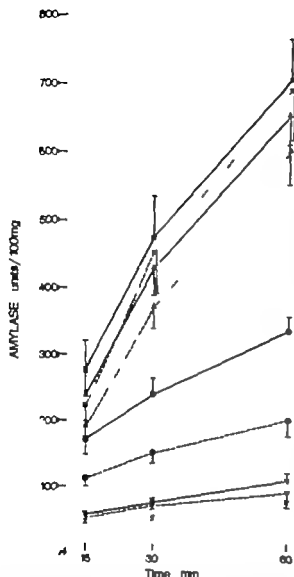


Fig. 1 Amylase secretion in response to different doses of isoprenaline alone (broken lines) and in the presence of carbachol in a concentration of 10^{-6} M (solid lines). Dotted line represents controls. The secretion is expressed as units of amylase released per 100 mg of parotid tissue. Isoprenaline was given in the following concentrations (M): 10^{-10} (∇), 10^{-6} (\bullet), 10^{-6} (Δ), 10^{-6} (\blacksquare). The observations with carbachol alone (10^{-6} M) did not differ from controls and have been omitted from the figure to make it plainer. They were as follows at the different time: 43 ± 6 , 54 ± 8 , 77 ± 11 units/100 mg. Each point of observation is the mean \pm S.E. of 7-9 expts.

glands the animals were anesthetized with urethane 2.0 g/kg given into a lateral ear vein. A tracheal cannula was inserted. The anesthesia made it possible to remove most of the fat and connective tissue from the gland in situ thereby minimizing the time between the removal of the gland and the start of the experiment. The glands were transferred to pre-gassed chilled (4°C) incubation medium which was allowed to slowly reach room temperature during further dissection. Instead of using tissue slices, fine lobules of a size of $1 \times 2 \times 2-3$ mm were cut out by follow-

ing the normal lobular anatomy of the gland. The procedure is likely to cause less damage to the acini than tissue slicing (Castle et al. 1971). Preliminary expts. showed that with this procedure the basal leakage of amylase during subsequent unstimulated incubation was low and that 90-95% of the original amount of the glandular amylase could be recovered from the tissue after incubation for 1 h.

After the final dissection samples of lobules were rapidly weighed and 45-55 mg of tissue was transferred to 25 ml Erlenmeyer flasks containing 10 ml of fresh medium. The incubation flasks were placed in a metabolic shake bath shaking at a frequency of 50 per minute and kept at a temperature of 37°C . After transfer to the bath the tissue was washed three times before stimulating or blocking agents were added. Washing was done by decanting medium and adding 10 ml of fresh medium at 5-10 min intervals. The medium was of Krebs-Ringer type and had the following composition (mM): NaCl 120, KCl 4.8, CaCl_2 2.6, MgCl_2 1.5, NaH $_{2}$ PO $_4$ 1.5, NaHCO_3 2.5. In addition the medium was fortified with glucose (1 mM) and β -hydroxybutyric acid (0.5 mM). The flasks were continuously gassed with a mixture of 95% O_2 and 5% CO_2 pH 7.40. After addition of stimulating drugs, samples of 0.1 ml were taken at 15, 30 and 60 min for amylase determination. Blocking drugs were added 10 min prior to stimulating drugs. All drugs were given in a volume of 0.1 ml.

For amylase determination a 0.1 ml sample was transferred to 0.9 ml phosphate buffer and then processed according to Dahlqvist (1964). In this method 1 unit of amylase is equivalent to the amount that liberates 10 reducing groups corresponding to 1 μmol of maltose formed each minute at 25°C . Tissue content of amylase was expressed as content in supernatants of spun tissue homogenates which were obtained by homogenizing incubated tissue in cold phosphate buffer immediately after the end of an expt.

Statistics

Student's *t*-test for paired observation was used. *P* values less than 0.05 were considered significant.

Drugs

Carbachol chloride, isoprenaline sulphate, propranolol (ICI), Hoechst 9980 (p-teridino-ethyl-diphenyl)-acetate, hydrochloride. All drugs were dissolved in physiological saline solution.

Results

Isoprenaline (10^{-10} M) produced a dose related increase of amylase secretion that could be further increased by the addition of carbachol in concentrations of 10^{-6} M. This effect on the isoprenaline evoked amylase secretion was particularly studied at a concentration of carbachol of 10^{-6} M, a carbachol concentration which was without own amylase secretory effect. The additional increase in

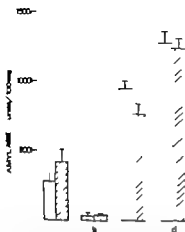


Fig. 2 Amylase depletion from parotid tissue after 60 min incubation with isoprenaline in a concentration of 10^{-6} M after alone (open columns) or in a combination with carbachol at a concentration of 10^{-6} M (hatched columns). Amylase secretion and tissue content is expressed as units per 100 mg of parotid tissue. Controls (not shown) released 47 ± 7 units/100 mg after 1.5 h incubation. Amylase release in the presence of agonists: (a) amylase release during additional 30 min incubation in absence of agonist before directly after exposure to agonists; (b) amylase remaining in tissue at end of incubation; (c) total amount of amylase (sum of a+b+c); d. Mean \pm S.E. from 4 experiments.

any release that carbachol brought about for each concentration of isoprenaline was maintained at an almost unchanged level throughout the experiments, which lasted for one hour (Fig. 1). The concentration of isoprenaline necessary to evoke amylase secretion was around 10^{-6} M which tended to increase the amylase release, however significantly only in one series of experiments (Fig. 3, $P < 0.05$). Fig. 1 shows that the presence of carbachol (10^{-6} M) regularly caused isoprenaline in this low concentration to release a significant amount of amylase after 30 ($P < 0.02$) and after 60 ($P < 0.01$) but not after 15 min incubation. The most pronounced effect of the addition of carbachol was seen at the isoprenaline concentration of 10^{-6} M: the amylase release by isoprenaline was increased two-fold at all three times of determination ($P < 0.001$). When the concentration of isoprenaline was increased to 10^{-5} M, carbachol could still promote an increase in amylase release by an additional 10% measured at 15, 30 and 60 min of incubation ($P < 0.02$). The amylase release in response to higher concentrations (10^{-4} M) was found to be maximal for isoprenaline and the amylase secretion was not further increased

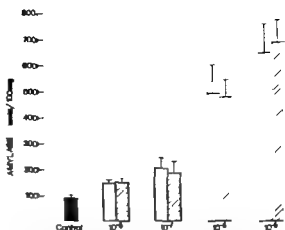


Fig. 3 Amylase secretion in response to 60 min incubation with isoprenaline (10^{-6} – 10^{-3} M) alone (open columns) and in a combination with carbachol (10^{-6} M, hatched columns). Black columns indicate controls. In all cases the atropine-like agent Hoechst 9980 (10^{-6} M) was added 10 min prior to agonists. The secretion is expressed as units released per 100 mg of parotid tissue. Mean \pm S.E. from 5 experiments.

by the addition of carbachol. The promoting effects of carbachol (10^{-6} M) on isoprenaline (10^{-6} M) evoked amylase release was also reflected in the amount of amylase that remained in the tissue after cessation of stimulation. Fig. 2 shows a significantly ($P < 0.05$) greater depletion of amylase in the lobules which in the presence of carbachol had secreted more amylase than in those which in the presence of isoprenaline only secreted less. In those experiments the 60 min of incubation with the stimulating agents was followed by an additional incubation period of 30 min in the absence of the drugs before the tissue content of amylase was determined. This was done to allow for any released amylase that could remain in the ducts to diffuse into the medium. For both conditions of incubation the amount of amylase released during the additional 30 min incubation and the total amount of amylase was the same. The promoting effect on the isoprenaline evoked amylase release that carbachol brought about was completely abolished by the atropine-like agent Hoechst 9980 which left the isoprenaline produced amylase secretion unaffected (Fig. 3).

The promoting effects of higher concentrations of carbachol (10^{-5} – 10^{-3} M) were studied at a certain concentration of isoprenaline (10^{-6} M). Concentrations of carbachol at 10^{-5} and 10^{-3} M produced a

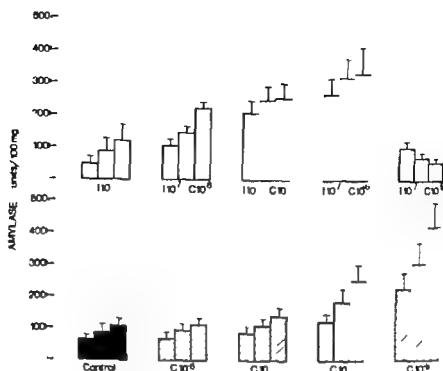


Fig. 4 The hatched column show amylase secretion in response to carbachol ($C 10^{-10}$ M). The amylase leakage from controls is shown as the black column. Above is shown the net amylase secretory effects by isoprenaline (10^{-10} M) alone and in the presence of carbachol of different concentrations. The net amylase secretory effect of isoprenaline has been obtained by subtracting the leakage in control plus the secretory effects by carbachol in paired experiments. The three columns represent 15, 30 and 60 min after addition of stimulating agents. The observations are the mean \pm S.E. from 5 expts.

continuous amylase secretion which was significantly larger than the leakage from controls ($P < 0.05$, $P < 0.02$ respectively Fig. 4). Carbachol

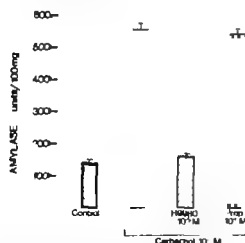


Fig. 5 Amylase secretion evoked by carbachol in a concentration of 10^{-4} M. The secretion of amylase is expressed as units secreted per 100 mg of parotid tissue. Black column show controls. Other columns show carbachol alone (open), in the presence of Hoechst 9980 (hatched) and in the presence of propranolol (dotted). Mean \pm S.E. from 5 expts.

10^{-7} and 10^{-6} M promoted isoprenaline to release larger amounts of amylase than carbachol 10^{-4} M ($P < 0.05$, $P < 0.02$ respectively) at 15 and 30 min but not after 60 min. Also the pattern of isoprenaline evoked amylase secretion changed to a more rapid release in the beginning of the incubation period instead of the gradual release seen in response to isoprenaline alone and to isoprenaline in the presence of carbachol in a concentration of 10^{-4} M. At these higher concentrations of carbachol isoprenaline released about 80% of its totally released amylase after 15 min and practically all of the remaining release occurred within the next 15 min. There was no difference between the promoting effects of carbachol at a concentration of 10^{-7} M and that of 10^{-4} M. Still higher concentrations of carbachol (10^{-4} M) regularly decreased the secretory effect of isoprenaline (10^{-7} M). The amylase secretory effect of carbachol could be completely blocked by the addition of Hoechst 9980 at a concentration of 10^{-4} M. Propranolol (10^{-4} M) did not affect the carbachol produced amylase secretion (Fig. 5).

DISCUSSION

The amylase secretory effects of isoprenaline in concentrations ranging from threshold to just sub-normal could all be increased in the presence of carbachol. The effect was particularly marked at low but suprathreshold concentrations of isoprenaline, at which the amylase secretory effect was increased by about 100%. The promoting effect on the isoprenaline produced amylase secretion was obtained both as an increased release of amylase into the medium and as a greater depletion of amylase from the tissue stores. This effect of carbachol was at all concentrations (10^{-6} – 10^{-4} M) abolished by the isoprenaline agent Hoechst 5940 (Schaumann & Linder 1961), used instead of atropine because of the possible occurrence of atropinase in rabbit tissue (Gronhaug & Bernheim 1938). It is suggested for the activation of muscarinic receptors may be in some way augmented the β -adrenoceptor mediated release of amylase. This assumption is supported by the great promoting effect that carbachol (10^{-4} M) had which did not produce any amylase secretion on its own. At this low concentrations of carbachol the augmenting effect was maintained throughout the experiment. The augmentation was obtained also at concentrations of 10^{-7} and 10^{-6} M but mainly during the first 30 min of observation. The change in promoting effect parallels with concentrations of carbachol that are known to release K⁺ from the rat parotid gland *in vitro* (Butcher et al 1976). Since K⁺ release *in vitro* is used as an indirect marker of fluid secretion *in vivo* (see Schaumann & Selinger 1976) the change in carbachol effect may have been due to the addition of fluid secretion to the augmentation of amylase secretion. In rabbit experiments similar to the present ones rather high concentrations of carbachol and isoprenaline (both at 10^{-6} M) in combination secreted more amylase after 5 min incubation than the sum of either alone (Wojcik, Grand & Kamberg 1975). In these expts. the main promoting effect of carbachol may have been due to rapid transport of amylase because of secretion of fluid. In expts. using superfusion techniques high concentrations of carbachol (4×10^{-6} M) or acetylcholine (10^{-6} M) have in mouse and rat parotid tissue been shown to transiently increase the amylase secretion in response to concentrations of isoprenaline of 10^{-6} – 10^{-4} M (Peterson & Ueda 1975; Harper & Brooker 1978), a result which was

attributed to fluid secretion. A cholinergic augmentation of β -adrenoceptor mediated fluid secretion similar to that demonstrated *in vivo* for the parotid gland of the rabbit (Gjörstrup 1977) may also have contributed to the promoting effects at low agonist concentrations. However the β -adrenoceptor mediated fluid secretion from the parotid gland *in vivo* is very sparse (Gjörstrup 1977) and isoprenaline *in vitro* generally regarded not to cause any h₂-release (Batzri et al 1973). The possibility of interactions at second messenger level between the two kinds of stimuli, that would favour secretion of amylase as it occurred in these expts. has been discussed (see Berridge 1975; see Rasmussen et al 1976). It has also been speculated about the possibility of a high parasympathetic activity to decrease the release of amylase by lowering the intracellular levels of cAMP (Butcher et al 1976; Harper & Brooker 1978; Oron et al 1978). In the present expts. an inhibition of isoprenaline evoked amylase secretion was seen at a concentration of carbachol of 10^{-7} M which alone produced an amylase secretion of the same magnitude as the highest one seen in response to isoprenaline. However the high amylase release does not correspond to the effects *in vivo* of parasympathetic nerve stimulation at physiological frequencies and may indicate that the effects of such high concentrations of carbachol are of little physiological significance (Asking & Gjörstrup 1980; Gjörstrup unpublished).

In the conscious rabbit the parasympathetic and sympathetic secretory nerves of the parotid gland were found to be simultaneously activated during feeding: the main function for the sympathetic nerves was to regulate the secretion of amylase (Gjörstrup 1979b). Judging from a comparison between the most commonly obtained values for the amylase output during feeding expts. and those values that were obtained in acute expts. with stimulation of the sympathetic nerves it seems as if the sympathetic activity during feeding is below 1 Hz for most of the time (Gjörstrup 1979). For low impulse traffic in the sympathetic nerves during feeding the presently described augmentation of amylase secretion may be of importance in the secretion of amylase. The regular response to a low concentration of isoprenaline (10^{-6} M) in the presence of carbachol is in accordance with the observations that very low thresholds were obtained *in vivo* for sympathetic stimulation (0.05 Hz) and isoprenaline injections (0.01 μ g/kg) in background of

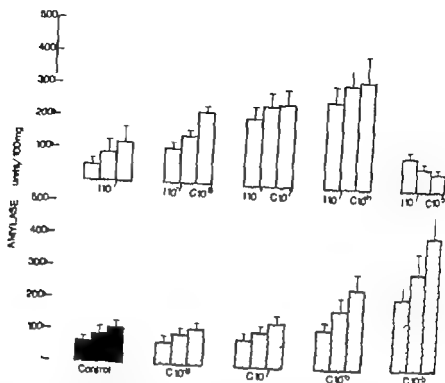


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continuous amylase secretion which was significantly larger than the leakage from controls ($P < 0.05$, $P < 0.02$ respectively) (Fig. 4). Carbachol

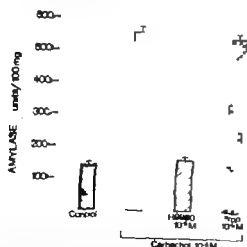


Fig. 5 Amylase secretion evoked by carbachol in a concentration of 10^{-6} M. The secretion of amylase is expressed as units secreted per 100 mg of parotid tissue. Black columns show controls. Other columns show carbachol alone (open), in the presence of Hoechst 9980 (hatched) and in the presence of propranolol (dotted). Mean \pm S.E. from 5 expts.

10^{-7} and 10^{-6} M promoted isoprenaline to release larger amounts of amylase than carbachol 10^{-10} ($P < 0.05$, $P < 0.02$ respectively) at 15 and 30 min but not after 60 min. Also the pattern of isoprenaline evoked amylase secretion changed to a more rapid release in the beginning of the incubation period instead of the gradual release seen in response to isoprenaline alone and to isoprenaline in the presence of carbachol in a concentration of 10^{-10} M. At these higher concentrations of carbachol isoprenaline released about 80% of its totally released amylase after 15 min and practically all of the remaining release occurred within the next 15 min. There was no difference between the promoting effects of carbachol at a concentration of 10^{-10} M and that of 10^{-6} M. Still higher concentrations of carbachol (10^{-6} M) regularly decreased the secretory effect of isoprenaline (10^{-6} M). The amylase secretory effect of carbachol could be completely blocked by the addition of Hoechst 9980 at a concentration of 10^{-6} M. Propranolol (10^{-6} M) did not affect the carbachol produced amylase secretion (Fig. 5).

Measurement of myometrial blood flow in rabbits by washout of Xenon 133 after atraumatic local labelling

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OTTESEN B. Measurement of myometrial blood flow in rabbits by washout of Xenon-133 after atraumatic local labelling. *Acta Physiol Scand* 1990; 109: 4: 1-126. Received 30 Nov. 1979. ISSN 0001-6772. Institute of Medical Physiology B, University of Copenhagen and Department of Clinical Chemistry, Hvidovre Hospital, Copenhagen, Denmark.

In vivo experiments were performed on the uterine tissue of non-pregnant estradiol treated rabbit anesthetized with sodium pentobarbitone. The uterine blood flow calculated from the washout of Xenon-133 after atraumatic labelling from the uterine surface was $25.4 \text{ ml} \cdot \text{min}^{-1} \cdot (100 \text{ g})^{-1}$ (range 7.4-46.6) and after local injection of the tracer in isotonic saline directly into the myometrium $4.4 \text{ ml} \cdot \text{min}^{-1} \cdot (100 \text{ g})^{-1}$ (range 3.7-45.3). During the experiment a monoexponential washout curve was found for the whole washout process both after atraumatic labelling and when the injected volume was $5 \mu\text{l}$. No trauma of injection was observed using this injection volume. The results support the applicability of a monoexponential model for the washout of inert gas from the myometrium. Thus myometrial blood flow can be calculated from the Xenon-133 washout rate at any time interval during the washout process.

Key words: Uterus, blood flow, ^{133}Xe , trauma of injection.

Uterine circulation has been measured in an animal by many different methods (Lewis 1969). The methods include collection of the venous effluent per time unit, estimation of uterine vascular resistance at constant flow, rotameter flowmeters, electromagnetic flowmeters and volume plethysmography. Other methods include applications of the Fick principle with N_2O or equilibration of ^3H -antipyrine, radioactive microspheres, heat conductivity measurement and isotope methods using Sodium-24, tritiated water and Xenon-133. The isotope method was introduced by Kety (1949) and applied to uterine muscle with Sodium-24 by Brown et al. (1963). Labelling with Xenon-133 of human skeletal muscle was introduced by Lassen et al. (1964). Monck et al. (1964) injected Xenon-133 dissolved in isotonic saline directly into the uterine muscle in 0.1-0.2 ml saline solution containing 50-100 μCi . This technique has subsequently been used widely both in animal experiments and in human investigation. Some authors report a dual compartment model for the non-pregnant myometrial blood flow (Forsman 1973); others

monocompartmental model (Jansson 1969). In other tissues including subcutaneous adipose tissue (Levin Nielsen 1977) and cutaneous tissue (Sejrtzen 1968) monoexponential washout curves have been obtained by an atraumatic labelling technique indicating a monocompartmental model. The aim of the present investigation is an attempt to measure blood flow in myometrial tissue from the washout curve of Xenon-133 following an atraumatic labelling and to compare the calculated values with those obtained using local injection of Xenon-133 dissolved in saline.

MATERIAL AND METHODS

The experiments were performed on female rabbit of the Danish Landrace weighing 400-3000 g, aged 140-170 days, and treated during eight days with daily intramuscular injections of $10 \mu\text{g}$ estradiol. The animals were fasted for 12 h and anesthetized with sodium pentobarbitone, 65 mg per kg b.wt. Tracheostomy was performed and the rectal temperature was kept at 37°C by thermostatized controlled table. A polyethylene catheter (Polystan, ID 0.76 mm, O.D. 1.22 mm) was inserted in the carotid artery for blood pressure registration and withdrawal of arterial

parasympathetic activity (Gjørstrup 1979a, Asking & Gjørstrup 1980).

This work was supported by grants to P. G. from the Medical Faculty, University of Lund.

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Measurement of myometrial blood flow in rabbits by washout of Xenon 133 after atraumatic local labelling

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OTTESEN B. Measurement of myometrial blood flow in rabbit by washout of Xenon-133 after atraumatic local labelling. *Acta Physiol Scand* 1980; 109: 41-426. Received 30 Nov 1979. ISSN 0001-6772. Institute of Medical Physiology B, University of Copenhagen and Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark.

In two experiments were performed on the uterine tissue of non-pregnant estradiol treated rabbit anesthetized with sodium pentobarbitone. The average blood flow calculated from the washout of Xenon-133 after atraumatic labelling from the uterine surface was 25.4 ml min⁻¹ (100 g)⁻¹ (range 7.4-66.6), and after local injection of the tracer in isotonic saline directly into the myometrium 4.4 ml min⁻¹ (100 g)⁻¹ (range 1.7-45.3). During the experiment monoexponential washout curves were found for the whole washout process both after atraumatic labelling and when the injected volume was 5 µl. No trauma of injection was observed using this injection volume. The results support the applicability of monoexponential model for the washout of inert gas from the myometrium. Thus myometrial blood flow can be calculated from the Xenon-133 washout rate at any time interval during the washout process.

Key words: Uterine blood flow, ¹³³Xenon, trauma of injection.

Uterine circulation has been measured in animals and man by many different methods (Lewis 1969). The methods include collection of the venous effluent per time unit, estimation of uterine vascular resistance at constant flow, rotameter flowmeters, electromagnetic flowmeters and volume plethysmography. Other methods include application of the Fick principle with N₂O or equilibration of ¹⁴C-antipyrine, radioactive microspheres, heat conductivity measurements and isotope methods using Sodium-24, tritiated water and Xenon-133. The isotope method was introduced by Kety (1949) and applied to uterine muscle with Sodium-24 by Jones et al. (1963). Labelling with Xenon-133 of uterine skeletal muscle was introduced by Lassen et al. (1964). Monick et al. (1964) injected Xenon-133 dissolved in isotonic saline directly into the uterine muscle as 0.1-0.2 ml saline solution containing 50-100 µCi. This technique has subsequently been used widely both in animal experiments and in clinical investigations. Some authors report a dual compartment model for the non-pregnant uterine blood flow (Forssman 1973), others a

monocompartmental model (Jansson 1969). In other tissues including subcutaneous adipose tissue (Levin Nielsen 1977) and cutaneous tissue (Sejrsen 1968) monoexponential washout curves have been obtained by an atraumatic labelling technique indicating a monocompartmental model. The aim of the present investigation is an attempt to measure blood flow in myometrial tissue from the washout curve of Xenon-133 following an atraumatic labelling and to compare the calculated values with those obtained using local injection of Xenon-133 dissolved in saline.

MATERIAL AND METHODS

The experiments were performed on female rabbits of the Danish Landrace weighing 400-3 900 g, aged 140-170 days, and treated during eight days with daily intramuscular injections of 25 µg estradiol. The animals were fasted for 1 h and anesthetized with sodium pentobarbitone, 65 mg per kg b.wt. Tracheostomy was performed and the rectal temperature was kept at 37°C by thermostatic controlled table. A polyethylene catheter (Polystan, I.D. 0.76 mm, O.D. 1.22 mm) was inserted in the carotid artery for blood pressure registration and withdrawal of arterial

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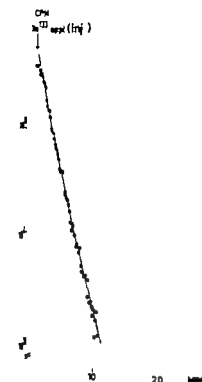


Fig. 4 The disappearance curve of Xenon-133 from the uterine horn after local intra-myometrial injection of 5 μ l Xenon-133. All adjacent tissue but the uterine horn is held off with lead sheets.

iodine (Tl) crystal 25 mm in diameter and 6 mm in thickness. The detector was placed about 5 cm above the surface of the uterus. The detector was coupled to a cable connection with preamplifier (Universal Printing Instrument, Mediscan). A pulse-height discriminator was used, adjusted around the 81 keV photopeak of Xenon-133. Measurement was started immediately after the

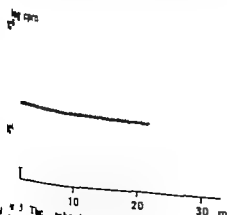


Fig. 5 The washout curve of Xenon-133 from uterine horn after intra-myometrial labelling. 5 min after the rabbit has been killed with an overdose of sodium pentobarbitone.



Fig. 6 The disappearance curve of Xenon-133 after intra-myometrial labelling during cooling followed by heating period.

isotope had been deposited and the results were printed out every 10 s. After subtraction of the background activity the results were plotted in semi-logarithmic diagrams, with the logarithm of the corrected count rate as ordinate and the time in minutes as abscissa.

Calculation

The washout of the isotope from the uterine horn was treated as a mono-compartmental model applying Kety (1949) classical mono-exponential clearance function

$$C(t) = C(0) \exp(-k_1 t) \exp(-t/\lambda)$$

leading to

$$MBF = \frac{\ln C(0)}{T_1} \lambda \quad (100 \text{ ml}/(100 \text{ g}) \cdot \text{min})$$

Where MBF is myometrial blood flow, T_1 is the half time of Xenon and λ is the tissue:blood partition coefficient which was chosen as assumed to be 0.7 ml/g in accordance with the figures found for skeletal muscle by Coen (1961) the tissue compositions of these two tissues are very similar (Kao 1967; Needham & Schoenberg 1967). $k = \ln 2/T_1$ is the rate constant of the washout process.

Experiment

In 11 experiments the myometrial blood flow was measured after intra-myometrial labelling. In 9 of these experiments additional flow measurements were performed after labelling

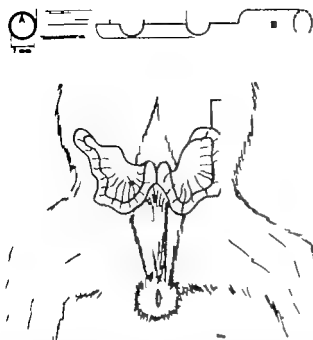


Fig. 1. Upper part: A split brass tube: transverse section (A) and longitudinal section (B). Two needles transfix the mesometrium and hold the uterus in the gutter. Lower part: Female rabbit with the uterus exposed and one of the uterine horns placed in the split brass tube.

blood samples. Arterial blood pressure was registered continuously with a transducer (Statham P 23 AA) connected to a recorder (Brush 220). Fluid replacement was performed with isotonic saline administered by an infusion pump (Braun) connected to the right external jugular vein via a polyethylene catheter.

The pH, $p\text{CO}_2$ and $p\text{O}_2$ in arterial blood were checked

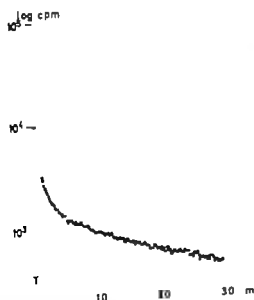


Fig. 2. The disappearance curve of Xenon-133 from the uterine tissue after atraumatic labelling without any collimation with lead sheets.

A 1 Ph and Sc and 109

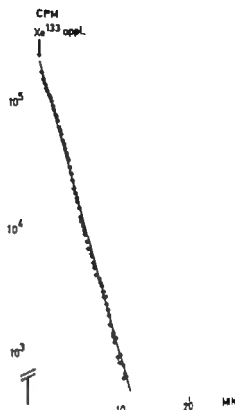


Fig. 3. Same registration as in Fig. 2. All tissue to uterine horn has been covered with lead sheets.

before each blood flow measurement by a Radi'vis blood micro system and pH/blood gas monitor. uterine horns were exposed through a midline laparotomy (Fig. 1). Part of a horn was fixed by placing it in a brass tube provided with two needles (Fig. 1) which forated the adjoining mesometrium in vessel-free area. The inner diameter of the tube chosen was the sum of the outer diameter of the uterine horn. This arrangement ensured a stable geometry during the registration of Xenon-133 washout. The temperature monitored by thermocouple placed near the surface of the myometrium was kept at 37°C by a heating lamp.

Labelling of the tissue

The atraumatic labelling of the uterine horn. With a micro syringe (1–50 μl Hamilton) 5 μl of Xenon-133 in hot saline solution (10–15 mCi/ml Kabi, Sweden) was deposited in the brass tube beneath the uterine horn. A fluid coat about 0.02 mm thick and covering an area about 2.2 cm^2 . The local intramyometrial injection of non-133 was performed with a needle (37G) mounted micro syringe (1–50 μl Hamilton). The needle was introduced almost parallel to the surface of the uterine horn and advanced 1 cm. 5 μl or less was injected and the needle very slowly redrawn to avoid leakage. The surface of the horn was covered with a gas-tight 20 μm thick Mylar membrane and moist gauze. Sheets of lead (3 mm thickness) were used to shield off all tissue except the area that located in the uterine horn. The lead sheet did not interfere with the blood supply. The Xenon-133 washout was measured by means of a scintillation detector.

Table 1. Values of uterine tissue blood flow gathered from the literature

| Author | Species | Hormonal state | Technique | Myometrial blood flow (ml/min (100 g)) mean values |
|-------------------------|-------------|----------------|--|---|
| McCall et al. (1959) | Sheep, goat | Pregnant | Nitrous oxide equilibration | 22.2 |
| McLachlan et al. (1961) | Goat | Pregnant | 4-Amino-antipyrine equilibration | 70.2 |
| Black et al. (1964) | Human | Pregnant | Washout after injection of Xenon-133 into the myometrium | 13 |
| | | Nonpregnant | Washout after injection of Xenon-133 into the porta | 11 |
| | | Nonpregnant | Washout after injection of Xenon-133 into the isthmus | 23 |
| Carter et al. (1968) | Rabbit | Pregnant | Washout after injection of Xenon-133 into the myometrium | 4 |
| Imeson (1969) | Human | Nonpregnant | Washout after injection of Xenon-133 into the myometrium | 3 |
| Imeson (1969) | Rabbit | Pregnant | ^{86}Sr and ^{14}C labelled microspheres | 14.5 |
| Johnson et al. (1975) | Rabbit | Pregnant | Drop counter system | 3.6 |
| | | | Ultrasonic doppler flowmeter | 27.1 |

(c) the tissue to blood partition coefficient for the indicator known

(d) the recirculation of Xenon-133 to the area seen by the detector is negligible

(e) only a minor influence from disappearance of Xenon-133 by other routes than the blood (leaking between the uterine horn and the brass tube and intercompartmental diffusion processes between myometrium and adjacent tissues) and

(f) only a negligible venous-arterial shunting by diffusion of Xenon-133 must take place

If these conditions are fulfilled blood flow becomes the only rate-limiting factor to disappearance of tracer from the labelled tissue and a monocompartmental model can be applied. It has previously been shown that for all practical purposes diffusion equilibrium can be obtained in the case of inert gas (Key 1949; Sejrsen & Tønnesen 1968). Constant and homogeneous blood flow can be obtained by keeping the experimental parameters constant: temperature, acid and base values, blood pressure etc. The partition coefficient has previously been found to be 0.7 in striated muscle (Conn 1961). This same and uterine myometrium are very similar in composition (Kao 1967; Needham & Schoenberg 1967) and thus have nearly identical partition coefficients. Recirculation is presumably negligible as judged from previous data (Tønnesen & Sejrsen 1970). Loss of Xenon-133 by diffusion through the narrow space between tissue and brass tube is presumably possible when atraumatic labelling is used. At the washout curves observed after atraumatic

labelling of the uterine horn have an almost mono-exponential form during the whole washout process which is in concordance with a monocompartmental model. In some expts. however there was a higher disappearance rate during 1-2 min probably due to initial leakage of the Xenon-133 through the narrow space between the uterine horn and the brass tube. Later on this had minor influence on the calculated blood flow value as concluded from smallness of the elimination rate observed after the animal had been sacrificed. The washout curves after labelling by local intramyometrial injection were also monoexponential. No trauma phase (Tønnesen & Sejrsen 1970) was found when the injected volume was 5 μl or less. Shunting by diffusion was of no significant importance because of the relatively high blood flow values in the present experiments (Sejrsen & Tønnesen 1977).

In Table 2 myometrial blood flow values have been gathered from the literature. There is no report on myometrial blood flow in non-pregnant rabbits but values from pregnant rabbits and other species are of the same magnitude as the present.

The measurement of uterine blood flow by atraumatic local labelling can be applied to small animals. It is possible to use local injection of the tracer. If the injected volume is sufficiently small to avoid significant changes in tissue composition in the depot area and elevation in interstitial fluid pressure leading to a decrease in the perfusion of the area.

The applicability of a monocompartmental model

Table 1 Myometrial blood flow (MBF) after atraumatic labelling and local intramyometrial injection

The pH, pCO₂, pO₂ and blood pressure are given for each measurement – not measured

| Rabbit No | Xenon-133 application | MBF (ml (100 g min ⁻¹)) | pH | pCO ₂ (mmHg) | pO ₂ (mmHg) | Blood pressure (mmHg) | | Weight of uterus (g) |
|-----------|-----------------------|-------------------------------------|-----|-------------------------|------------------------|-----------------------|-----------|----------------------|
| | | | | | | Systolic | Diastolic | |
| 1 | Atraumatic | 9.1 | 7.5 | 38.3 | 74 | 110 | 76 | 6.7 |
| | Atraumatic | 51.8 | 7.3 | 35.2 | 94 | 111 | 86 | 6.1 |
| | Injection | 34.7 | 7.4 | 3.3 | 99 | 120 | 90 | 6.1 |
| 3 | Atraumatic | 18.2 | 7.4 | 34.3 | 101 | 133 | 100 | 12.0 |
| | Injection | 20 | 7.5 | 30.3 | 104 | 116 | 90 | 12.0 |
| 4 | Injection | 26.3 | 7.5 | 28.8 | 95 | 113 | 86 | 1.5 |
| | Atraumatic | 29.1 | 7.5 | 5.6 | 100 | 113 | 86 | 1.5 |
| 5 | Atraumatic | 18 | 7.4 | 4.8 | 97 | 106 | 73 | 7.2 |
| | Injection | 15.7 | 7.4 | 38.0 | 68 | 100 | 73 | 7.2 |
| 6 | Injection | 4.4 | 7.5 | 3.2 | 83 | 120 | 90 | 9.9 |
| | Atraumatic | 5.4 | — | 77.8 | 75 | 100 | 80 | 9.9 |
| 7 | Atraumatic | 39.8 | — | 4.7 | 79 | 120 | 90 | — |
| | Injection | 20.1 | 7.5 | 35.5 | 75 | 126 | 86 | — |
| 8 | Injection | 8.7 | 7.4 | 36.6 | 83 | 100 | 73 | 7.6 |
| | Atraumatic | 7.4 | 7.4 | 41.2 | 71 | 106 | 73 | 7.6 |
| 9 | Atraumatic | 1.8 | 7.4 | 40.5 | 78 | 100 | 80 | 8.1 |
| | Injection | 47.9 | — | 40.0 | 80 | 104 | 70 | 9.0 |
| 10 | Atraumatic | 45.3 | — | 41.2 | 79 | 110 | 80 | 9.0 |
| | Injection | 5.3 | 7.4 | 37.2 | 100 | 11 | 90 | — |
| 11 | Atraumatic | 66.6 | — | 35 | 74 | 110 | 80 | — |

by direct injection of 5 µl Xenon-133 in isotonic saline into the myometrium. In *expts.* the temperature of the uterine tissue was varied by means of cooling (icebags) and heating (heating table and lamp). Each experiment was terminated with a period of measurement after the animal had been sacrificed with an overdose of sodium pentobarbitone.

RESULTS

A representative Xenon 133 washout curve after atraumatic labelling without covering adjacent tissue with lead sheets is shown in Fig. 2. This curve can be divided into 2 exponentials. A similar biexponential washout curve is registered after shielding off the uterine horn (not shown). Fig. 3 shows a washout curve after shielding off all tissue but the uterine horn. The latter disappearance curve of tracer can be fitted by a straight line over more than 2 decades in a semilogarithmic diagram ($r=0.999$, $P<0.001$). During the first 2 min the slope seems to be a little steeper, possibly due to leakage by diffusion of Xenon 133 out between the uterine horn and the brass tube. The same monoexponential washout curve was seen after direct injection of 5 µl Xenon-133 into the myometrium (Fig. 4). In preliminary *expts.* a larger volume (50–150 µl) of tracer solution was injected locally, but this yielded biexponential curves, probably due to trauma of injection.

The elimination rate measured after the animal had been sacrificed (Fig. 5) corresponds to less than 1.5 ml min⁻¹ (100 g) given as blood flow. The alterations in blood flow accompanying temperature variations are shown in Fig. 6. During the period of cooling the blood flow decreases gradually, and the following heating period results in increasing blood flow values caused by vasodilatation. Values for blood flow, pH, pCO₂, pO₂, blood pressure and weight of uterus are shown in Table 1.

The average flow value after atraumatic labelling was 25.4 ml min⁻¹ (100 g) (range 7.4–66.6) and after local myometrial injection 4.4 ml min⁻¹ (100 g) (range 8.7–45.3). No significant difference between the results obtained with the two methods was found (paired *t*-test).

DISCUSSION

The Xenon 133 washout technique gives reliable values for blood flow only if the following conditions are fulfilled:

- (a) diffusion equilibrium between tissue and blood is obtained during the whole washout
- (b) a constant and homogeneous distribution of blood perfusion is present

Table 1. Values of uterine tissue blood flow g derived from the literature

| Author | Species | Hormonal state | Technique | Myometrial blood flow (ml/min (100 g)) mean values |
|---------------------------|-------------|----------------|--|--|
| Leitch et al. (1959) | Sheep, goat | Pregnant | Nitrogen-vide equilibration | 22.4 |
| McLabee III et al. (1961) | Goat | Pregnant | 4-Amino-antipyrine equilibration | 20.4 |
| Black et al. (1964) | Human | Pregnant | Washout after injection of Xenon-133 into the myometrium | 13 |
| | | Nonpregnant | Washout after injection of Xenon-133 into the portio | 11 |
| | | Nonpregnant | Washout after injection of Xenon-133 into the isthmus | 3 |
| Carter et al. (1968) | Rabbit | Pregnant | Washout after injection of Xenon-133 into the myometrium | 4 |
| Samson (1969) | Human | Nonpregnant | Washout after injection of Xenon-133 into the myometrium | 23 |
| Tham (1969) | Rabbit | Pregnant | ^{86}Sr and ^{14}C labelled microspheres | 14.5 |
| Salomon et al. (1975) | Rabbit | Pregnant | Drop counter system | 23.6 |
| | | | Ultrasonic doppler flow meter | 77.1 |

(c) the tissue to blood partition coefficient for the tracer is known,

(d) the recirculation of Xenon-133 to the area seen by the detector is negligible

(e) only a minor influence from disappearance of Xenon-133 by other routes than the blood (leaking between the uterine horn and the brass tube, and intercompartmental diffusion processes between myometrium and adjacent tissues) and

(f) only a negligible venous-arterial shunting by leakage of Xenon-133 must take place

If these conditions are fulfilled blood flow becomes the only rate-limiting factor to disappearance of tracer from the labelled tissue and a mono-compartmental model can be applied. It has previously been shown that for all practical purposes diffusion can be obtained in the case of inert gas

Kety 1949; Sejersten & Tønnesen 1968). Constant and homogeneous blood flow can be obtained by keeping the experimental parameters constant: temperature, acid and base values, blood pressure etc. The partition coefficient has previously been found to be 0.7 in striated muscle (Conn 1961). This value and uterine myometrium are very similar in composition (Kuo 1967; Needham & Schoenberg 1967) and thus have nearly identical partition coefficients. Recirculation is presumably negligible as judged from previous data (Tønnesen & Sejersten 1967). Loss of Xenon-133 by diffusion through the narrow space between tissue and brass tube is practically possible when airtight labelling is used. All the washout curves observed after airtight

labelling of the uterine horn have an almost mono-exponential form during the whole washout process which is in concordance with a mono-compartmental model. In some experiments, however, there was a higher disappearance rate during 1-2 min probably due to initial leakage of the Xenon-133 through the narrow space between the uterine horn and the brass tube. Later on this had minor influence on the calculated blood flow value as concluded from smallness of the elimination rate observed after the animal had been sacrificed. The washout curves after labelling by local intramyometrial injection were also mono-exponential. No transient phase (Tønnesen & Sejersten 1970) was found when the injected volume was 5 μ l or less. Shunting by diffusion was of no significant importance because of the relatively high blood flow values in the present experiments (Sejersten & Tønnesen 1971).

In Table 2 myometrial blood flow values have been gathered from the literature. There is no report on myometrial blood flow in non-pregnant rabbits but values from pregnant rabbits and other species are of the same magnitude as the present.

The measurement of uterine blood flow by airtight local labelling can be applied to small animals. It is possible to use local injection of the tracer if the injected volume is sufficiently small to avoid significant changes in tissue composition in the depot area and elevation in interstitial fluid pressure leading to a decrease in the perfusion of the area.

The applicability of a mono-compartmental model

for the washout process of inert gas from the myometrial tissue enables us to calculate an average myometrial blood flow at any time interval during the period of washout. Thus any changes in average blood flow from one period to another can be calculated from the washout curves.

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Transient vasopressin release and thirst in response to prolonged intracerebroventricular infusions of hypertonic mannitol in saline

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MCKINLEY M. J., OLSSON K., FYHRQUIST F. & LILJEKVIST E. Transient vasopressin release and thirst in response to prolonged intracerebroventricular infusions of hypertonic mannitol in saline. *Acta Physiol Scand* 1980; 109: 427-431. Received 3 Dec. 1979. ISSN 0001-6772. Department of Animal Physiology, Swedish University of Agricultural Sciences, Uppsala, Sweden and the Manner Institute for Medical Research, Helsinki, Finland.

In the conscious goat infusions of 0.4 M mannitol in 0.15 M NaCl into the lateral cerebral ventricle (40 or 100 µl min⁻¹, 0.02 ml/min) caused slight, transient vasopressin release and temporary thirst. Whereas infusions of pure hypertonic (0.7 M) mannitol did not elicit thirst and inhibited the basic vasopressin release in the nonhydrated animal. In contrast, infusions of equiosmolar (0.35 M) NaCl induced persistent thirst and pronounced elevation of the plasma vasopressin concentration throughout the infusion period. The cerebrospinal fluid (CSF) osmolality was raised by the same order of magnitude ($\approx 13\%$) after the mannitol/NaCl and the hypertonic NaCl infusions. The CSF Na⁺ concentration was elevated by $>10\%$ at 5 min after the hypertonic NaCl infusions, but it was reduced by approximately 10% at 5 min after the mannitol/NaCl infusions. There was no appreciable difference in the CSF K⁺ concentration after the infusions. The results are discussed with regard to the possible importance of CSF Na⁺ concentration as opposed to strict osmotic factors for the activation of receptors involved in the control of water balance.

Key words: intracerebroventricular mannitol/NaCl, thirst, vasopressin antidiuresis, cerebrospinal fluid, goat.

The question of whether cerebral receptors involved in the control of water balance primarily are osmotic- or osmosensitive remains controversial (cf. Valerius 1978). It has recently been suggested, however, that receptors of both kinds may participate in the regulation of vasopressin secretion and water intake (McKinley, Denton & Weninger 1979). This resulted from comparison of the responses in sheep to intracerebroventricular (ICV) administration of hypertonic NaCl with those to equiosmolar sucrose and fructose dissolved in artificial cerebrospinal fluid (CSF). A previously reported lack of antidiuretic and dipsogenic responses to ICV administration of pure, hypertonic sucrose solution (Olsson 1969) was confirmed, whereas ICV sucrose and fructose dissolved in artificial CSF were found to cause antidiuresis and some drinking.

However, the antidiuretic and dipsogenic effects were considerably weaker than those obtained in response to equiosmolar NaCl. These experiments in sheep supporting a dual osmoreceptor/sodium sensor mechanism involved ICV injections or brief (10 min) infusions, and the vasopressin response was assessed indirectly by studying the degree of inhibition of the water diuresis of hydrated animals. It was therefore of interest to reproduce the experiments in the goat using prolonged ICV infusions combined with radioimmunoassay determination of blood plasma arginine vasopressin (AVP). In the present experiments mannitol was substituted for sucrose and fructose since mannitol appar-

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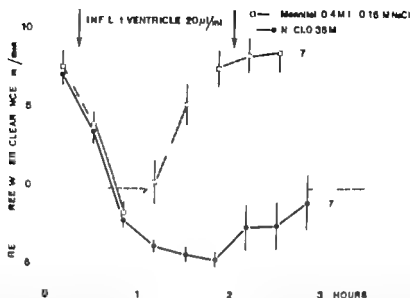


Fig. 1. Marked and persistent antidiuretic response to the ICV infusion of 0.35 M NaCl (dots) as opposed to the weak and transient response to similar infusions of 0.4 M mannitol in 0.15 M NaCl. C_{R, H₂O} = renal free water clearance. The symbol represent mean and the bars S.E.

ently is equally potent as an osmotic stimulus (Szczepanska-Sadowska & Koslowski 1975).

METHODS

Animal. Five female goats were used (b.wts. 35–55 kg). They were routinely kept in metabolism cages and were fed chopped hay three times daily. Each afternoon the animals received 200 g of commercial grain mix with 6 g of NaCl added.

Experimental procedure. All goats were provided with a permanent platinum-iridium cannula implanted into one of the lateral cerebral ventricles as described previously (Åkертund Andersson & Olsson 1973). ICV infusion were made at the rate of 0.02 ml/min for 40 or 100 min periods. Mannitol (0.4 M) was dissolved in 0.15 M (isotonic) NaCl and in most experiments K, Mg and Ca were added to the solution in normal CSF proportions. A pure 0.7 M solution of mannitol was used for ICV infusions in one series of experiment. The comparative experiments involved the ICV infusion of equiosmolar (0.35 M) NaCl. In hydration experiments the goats were given 100 ml/kg b.wt. of warm water by stomach tube into the rumen about 90 min before start of the ICV infusion. In experiments involving studies of the thirst response animals displaying an urge to drink were prevented from consuming significant amounts of water during the initial part of the infusion period. Thus when an animal became thirsty during an infusion a bucket was put to cover the water bucket and the goat did not obtain free access to water again until the ICV infusion had proceeded for 30 min.

Samples. For collection of CSF a polyethylene tubing was attached to the ICV cannula with its free outlet held lower than the skull. The head of the animal was slightly bent downwards with the effect that CSF dripped out of

the tubing. After discarding the initial three drops, the 0.3 ml of CSF was collected. Blood samples were taken via a polyethylene cannula introduced into the jugular vein before the experiment. Samples for analyses of plasma AVP were collected in chilled tubes with 0.3 M Na-EDTA as a anticoagulant and were immediately centrifuged at +4°C. The plasma was stored at -20°C until radioimmunoassayed. Urine was collected in 20 min samples via a retention catheter introduced into the urinary bladder.

Analytical method. Determination of plasma AVP were made according to Fyhrquist et al. (1976). CSF Na and K concentrations were measured by internal standard flame photometry using an IL 343 flame photometer. An Adv. Instruments Inc. osmometer was used for determination of the osmolality of the CSF.

Statistics. Values in both text and figures are expressed as mean and S.E. Student's *t*-test was employed for statistical analyses.

RESULTS

Antidiuretic effect in hydrated animals

ICV infusions (100 min) of 0.4 M mannitol in 0.15 M NaCl (*N* = 7) were made in the pre-hydrated goat (animals). In five of the experiments K, Mg and Ca were added to the infusions in normal CSF proportions. The infusions rapidly reduced the renal free water clearance (C_{R, H₂O}) which became negative within 40 min. The antidiuretic effect was transient however. Thus the water diuresis returned to pre-infusion level before the end of the infusion period (Fig. 1 squares). In contrast corresponding

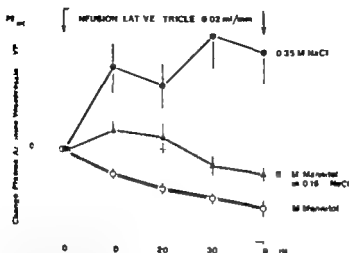


Fig. 2 Persistent increase in plasma vasopressin (AVP) concentration during ICV infusion of 0.35 M NaCl. Triangles: Slight increase of plasma AVP followed by a fall below the pre-infusion level during the ICV infusions of 0.4 M mannitol in 0.15 M NaCl. Circles: Progressive fall in plasma AVP concentration induced by the ICV infusions of 0.7 M mannitol. The pre-infusion plasma AVP concentration was 3.7 ± 0.4 pg/ml ($n = 20$). The symbols represent mean and the bars S.E.

ICV infusions of 0.35 M NaCl ($N=7$) induced a long-lasting inhibition of the water diuresis with renal C_{H_2O} remaining negative throughout the infusion period and for at least 60 min thereafter (Fig. 1 (b)).

Infusion on plasma vasopressin concentration

Plasma AVP was determined at 10 min intervals before and during 40 min ICV infusions in non-hydrated goats (4 animals). The study involved infusions of 0.4 M mannitol in 0.15 M NaCl ($N=9$) of 0.7 M mannitol ($N=4$) and of 0.35 M NaCl ($N=7$). In 4 of the mannitol/saline and 4 of the NaCl experiments K⁺, Mg²⁺ and Ca²⁺ were added to the solutions in normal CSF proportions. The mean pre-infusion plasma AVP concentrations were before infusions of mannitol/NaCl 4.4 ± 0.6 pg/ml before infusions of mannitol/H₂O 4.1 ± 0.9 pg/ml and before infusions of NaCl 2.5 ± 0.5 pg/ml. Since for methodological reasons, the pre-infusion concentrations varied, the induced changes from pre-infusion level are presented in Fig. 2. As evident from the figure the infusion of 0.35 M NaCl caused a marked and persistent rise in the plasma AVP (Fig. 2, triangles), increase over pre-infusion level at termination of the infusion, $P < 0.01$. A transient increase in plasma AVP was observed during the mannitol/

saline infusions, but here the AVP values returned to or fell below pre-infusion level within 30 min (Fig. 2, triangles). A progressive fall in plasma AVP was seen during infusions of 0.7 M mannitol (Fig. 2, circles), reduction from pre-infusion level at termination of infusion $P < 0.05$.

Thirst

The dipsogenic response to 40 min ICV infusions of 0.4 M mannitol in 0.15 M NaCl ($N=7$) was studied in 3 goats having had free access to water until the infusions were started. The mannitol/NaCl solution contained K⁺, Mg²⁺, Ca²⁺ in normal CSF proportions. For comparison the dipsogenic response to similar infusions of equimolar (0.35 M) NaCl ($N=7$) was studied in the same animals. In 3 of the mannitol/NaCl experiments no urge to drink developed during the infusion. In the remaining 4 experiments the animals attempted to drink 5 to 9 min after start of the infusion but were prevented from drinking until the infusion had proceeded for 30 min. In all but 1 experiment the urge to drink had vanished at this stage. In the exceptional experiment the goat drank 200 ml of water. A much stronger and persistent thirst response was obtained during the corresponding ICV infusions of equimolar 0.35 M NaCl. In all these experiments thirst became apparent 4 to 8 min after the start of the infusion, and the goats drank

Table 1 Changes in CSF osmolality, Na⁺ and K⁺ concentrations 5 and 70 min after 40 min ICV infusions of 0.4 M mannitol in 0.15 M NaCl (N=6) or 0.35 M NaCl (N=7)

The rate of infusion was 0.07 ml/min. Mean and S.E. are shown

| | Pre-infusion | 5 min post-infusion | | 70 min post-infusion | |
|------------------------|--------------|---------------------|---------|----------------------|---------|
| | | Man/NaCl | NaCl | Man/NaCl | NaCl |
| Osmolality mosm/kg | 294± | 336±7 | 379±5 | 308±3 | 304±2 |
| Na ⁺ mmol/l | 150±1 | 134± | 167± | 143±1 | 144±1 |
| K ⁺ mmol/l | 8±0.0 | 6±0.0 | 2.8±0.0 | 7±0.0 | 2.7±0.0 |

830±170 ml of water when obtaining free access to water after 30 min of infusion.

Changes in CSF composition

Samples of CSF were taken before and after (5 and 70 min) the 40 min ICV infusions of mannitol/NaCl (N=6) and NaCl (N=7) in three animals. The results of the subsequent determinations of osmolality [Na⁺] and [K⁺] are presented in Table 1. As shown in this table, the rise in CSF osmolality observed 5 min after the infusion was not significantly different between the mannitol/NaCl and the NaCl expts. As expected the [Na⁺] was considerably elevated (>10%) 5 min after the infusions of 0.35 M NaCl. However at the same stage it was lowered by about 10% in the mannitol/NaCl expts. It was still considerably below pre-infusion level 15 min later. A 0.2 mM reduction of CSF K⁺ was observed 5 min after the mannitol/NaCl infusions.

DISCUSSION

It was originally suggested by Andersson (1971) that cerebral receptors involved in the regulation of vasopressin secretion and water intake may be sodium sensitive rather than osmosensitive (Verney 1947) and that the receptors may have a juxta-ventricular location which makes them easily accessible to stimulatory and inhibitory influences exerted by the CSF composition. The suggestion was based on two observations made in the goat: (a) that hypertonic sucrose (in contrast to hypertonic NaCl) did not elicit water intake and apparent vasopressin release when infused into the third cerebral ventricle (Olsson 1969) and (b) that the diuretic and antidiuretic responses to similar infusions of angiotensin II were positively correlated to the CSF [Na⁺] (Andersson, Eriksson & Olner 1970). Since then the experimental evidence has become con-

siderably more substantiated that juxta-ventricular sodium sensitive receptors are involved in the control of water balance. ICV infusions of iso- or hypertonic saccharide solutions were found to inhibit the basal vasopressin secretion in non-hydrated normovolemic goats (Eriksson 1974, Olsson et al. 1978, Eryhquist, Eriksson & Wallenar 1979) and to attenuate or extinguish the urge to drink in dehydrated animals (Olsson, Larsson & Liljekvist 1976). Inhibition of vasopressin release with ICV infusion of saccharide solutions was obtained also when cations other than Na⁺ were added to the infusion in normal CSF proportions, but not when the saccharides were dissolved in isotonic saline. This suggested that ICV administration of pure non-electrolyte solutions induced negative water balance primarily by way of a dilutional reduction of the prevailing CSF [Na⁺] (Eriksson 1976).

The present study in goats confirms observations made in sheep (McKinley, Blaine & Denton 1974, McKinley et al. 1978) that CSF hyperosmolality induced by saccharides remaining in the extracellular space elicits thirst and vasopressin release if a reduction of the CSF [Na⁺] is temporarily prevented by the simultaneous ICV administration of NaCl. Therefore the present results may be regarded as additional support for the suggestion that cerebral osmoreceptors and sodium sensors interact in the control of water balance (McKinley et al. 1978). Also the facts that the urge to drink vanished and the plasma vasopressin returned to or fell below pre-infusion levels (Fig. 1) during prolonged ICV infusions of mannitol/NaCl appear compatible with the dual osmoreceptor/sodium sensor concept. The pronounced decrease in CSF [Na⁺] observed soon after the mannitol/NaCl infusion (Table 1) suggested that the disappearance of thirst and the reversal of the AVP response oc-

and concomitantly with a progressive reduction of the CSF $[\text{Na}^+]$. Most likely this fall in CSF $[\text{Na}^+]$ is due to mannitol-induced osmotic movement of water from the surrounding brain tissue and to blood. However the pronounced post-infusion CSF hyperosmolality (Table 1) indicates that the stimulation of water to the CSF during the infusion period is not sufficient to eliminate the osmotic component of a stimulus for a tentative osmoreceptor-mediated sensor mechanism. Nevertheless the antidiuretic (Fig. 1), AVP (Fig. 2) and dipsogenic responses gradually vanished during the infusion period. Therefore, if these responses were due to stimulation of osmoreceptors in Verney's (1947) sense, it appears that the excitation mechanism of the osmoreceptors was counteracted by the effect of abnormal extracellular $[\text{Na}^+]$.

A comparison between the immediate (5 min) post-infusion CSF osmolality values (Table 1) indicates that the strict osmotic stimulus component was of approximately the same magnitude during the ICV infusions of hypertonic NaCl and mannitol/ NaCl . In spite of this the NaCl infusion acted as a much more efficient and persistent stimulus of thirst and AVP secretion, which also may be regarded compatible with the dual osmoreceptor/sodium sensor concept. However whether the osmotic and Na^+ stimuli act at different cells in the brain is not clear from the present experiments. The results may possibly be regarded also as support for a theory advocating a more uniform character of cerebral receptors subserving water economy. Effects obtained in response to ICV administration of inhibitors and stimulators of enzymatic transmembrane Na^+ -transport hint at the possibility that enzymatic activity of this kind may be of crucial importance for the excitation of juxta-ventricular receptors regulating vasopressin secretion and water intake (cf. Andersson 1978). It led Andersson (1978) to suggest that elevated Na^+ concentration and cellular dehydration both may trigger a biochemical process that constitutes an essential link in the receptor excitation mechanism.

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Early effects of cervical sympathetic stimulation on cerebral, ocular and cochlear blood flow

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BEAUSANG-LINDER, M & HULTCRANTZ, E. Early effects of cervical sympathetic stimulation on cerebral, ocular and cochlear blood flow. *Acta Physiol Scand* 1980 109: 433-437. Received 5 Dec. 1978. ISSN 0001-6772. Department of physiology and Medical Biophysics, Biomedical Centre, Uppsala University and Department of Otolaryngology, University Hospital, Uppsala, Sweden.

Autoregulatory mechanisms may be expected to modify effects of vasomotor nerve stimulation in many tissues. An attempt was made to reveal distinct early but transient effects of cervical sympathetic stimulation on cerebral, retinal and cochlear blood flow. The labelled microsphere method was used to determine regional blood flow during electrical stimulation of the cervical sympathetic chain for 15-25 and 5 min. At frequency of 6 Hz there was 5% reduction in cerebral flow at 15-25 and 7% at 5 min. In the choroid plexus the mean reduction was 22% at 15-25 but decreased to 10% after 5 min. In the cerebellum, optic nerve and retina, sympathetic stimulation had no appreciable effect on the blood flow. In the cochlea and iris, the blood flow reductions were 25 and 32% respectively on both occasions. In the choroid, vasoconstriction increased with time whereas in the masseter muscle there was decrease. Thus in the present experiments no indication was found of an autoregulatory escape phenomenon in the brain, the eye or the cochlea. Some escape was noted in the masseter muscle.

Key words: Cerebral, ocular, cochlear blood flow/cervical sympathetic stimulation/microspheres.

The effect of sympathetic stimulation on the cerebral blood flow (CBF) has not been conclusively clarified. Some widely different results have been published (Harper et al. 1972, Heistad et al. 1977, Sorense et al. 1975, Traystman & Rapela 1975). Using the labelled microsphere method Alm & Brill (1977) found little or no effect of stimulation at 1 min or at 15-30 min. More recently Lacombe et al. (1977) reported that sympathetic stimulation induced flow decreases of 12-29% according to structures in the brain, using the ethanol tissue sampling method in non-anesthetized rabbits 15-30 s after stimulation started. This indicated a possibility of an early transient effect on the cerebral blood flow followed by an autoregulatory adjustment. The purpose of the present investigation was to study the effect of unilateral sympathetic stimulation on the blood flow to the brain, the eye and the cochlea in young rabbits using the labelled microsphere method (Griffin & Lindseth 1958, Wagner et al. 1969, Alm & Brill 1977) to determine whether there

was any difference between the effect after 15-30 s of stimulation and that after 5 min.

MATERIAL AND METHODS

Ten young rabbits of both sexes weighing between 1.4-3.0 kg were used. The animals were anesthetized with pentobarbital sodium 60 mg/kg, tracheotomized and ventilated by Palmer pump. They were placed on a servocontrolled heating pad and had temperatures around 38°C. Both femoral arteries were cannulated, one for continuous blood pressure measurements and the other for blood sampling. Both sympathetic cervical trunks and both vagal nerves were identified and cut above the thoracic aperture. The sympathetic nerve on one side was carefully prepared, connected to a bipolar silver electrode isolated from the surroundings to avoid stimulation of other nerves. The stimulation frequency was 6 Hz, intensity 7 V and duration 2 min. The effect was judged by the pupillary response. The frequency 6 Hz is supposed to exist physiologically under moderate to intense stress conditions (Mellander & Johansson 1966) and produces almost maximal pupil dilatation. After thoracotomy and control of the acid-base balance the stimulation was started. After 15 s of stimulation the first dose of microspheres was

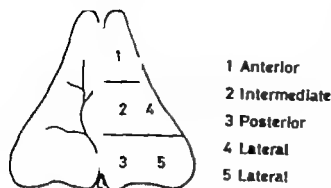


Fig. 1. Dissection of the brain into the different parts.

injected into the left ventricle of the heart and the blood sampling was started (six 10-second samples) from free flow of the femoral artery at the same time. The injection time was about 10 s and the sampling time always exactly 60 s. The stimulation was continued and after another 5 min the second injection of microspheres was made using differently labelled microspheres. Blood was sampled for 60 s and the animal was put to death by intracardial injection of KCl.

The brain, the eyes, the membranous parts of the cochleas (Angelborg et al. 1977) and samples of the masseter muscles were dissected out. The blood samples were weighed and the radioactivity was determined with a gamma spectrometer. The blood flow was calculated using the formula

$$\frac{f_i}{f_R} = \frac{f_{ref}}{n_{ref}}$$

where f = blood flow in the tissue g min⁻¹, f_{ref} = the reference blood flow g min⁻¹, n_i = radioactivity of the tissue and n_{ref} = radioactivity of the reference blood sample. The regional blood flow on the stimulated and non-stimulated sides were compared. Each hemisphere of the brain was dissected into 5 parts representing anterior, posterior, intermediate and two lateral regions (Fig. 1). The ciliary body was separated from the iris. About 7 mm of the distal part of the optic nerve was taken as one sample.

Microsphere

The microspheres used were $9 \pm 0.9 \mu\text{m}$, $8.7 \pm 0.6 \mu\text{m}$ and $15 \pm 1.0 \mu\text{m}$ in diameter and labelled with ¹⁴C and ⁸⁵Sr (3 M Co St Paul Minnesota). In 7 experiments both measurements were made with small spheres in a number of about 17×10^6 in each injection. In 3 experiments the first injection consisted of spheres $15 \mu\text{m}$ in diameter in a number of 4×10^6 and the second injection of 17×10^6 spheres with $8.7 \mu\text{m}$ in diameter. Large amounts of spheres were used to get a sufficient number into the cochlea (Buckberg et al. 1971). The experiments with two sizes of spheres were performed to check whether the results were dependent on the size of the spheres used. The spheres were suspended in saline and 10% Dextran aggregates were di-

rupted using a Potterhelim homogeniser. Each injection was 1.5–2 ml.

RESULTS

All the animals tolerated both the anaesthesia and the surgical manipulation well. The mean arterial blood pressure and values for the acid-base balance are presented in Table 1. In the 3 animals where $15 \mu\text{m}$ spheres were used in the first injection the blood pressure was unstable during the first 10 s but the volume of the blood samples collected during these 10 s was similar to the subsequent samples. During the second injection the blood pressure was stable. Mydriasis was near maximal within 15 s of sympathetic stimulation.

Values for the mean blood flow in the different tissues are presented in Tables 2 and 3.

Paired comparisons between the blood flow on the stimulated and non-stimulated side were performed in each experiment and the differences were expressed as percentages of the flow of the non-stimulated side. During the first measurement the mean difference \pm S.E. between the two hemispheres of the brain was $5.0 \pm 1.1\%$. This difference was significant ($P < 0.01$) using Student's t -test for paired comparisons. During the second measurement the difference between the stimulated and the control side was $7.0 \pm 5.5\%$ ($P < 0.01$). During both injections the blood flow was about 15% higher in the posterior part of the brain than in the anterior part (Fig. 1) ($P < 0.05$ using the t -test for paired comparisons).

In the anterior part no significant difference in blood flow was seen between the stimulated and the

Table 1. Acid-base values before and mean arterial blood pressure during the blood flow measurements.

| Exp no | MAP cm H ₂ O | | pH | P (kPa) |
|--------|-------------------------|-------|------|---------|
| | Inj 1 | Inj 2 | | |
| 1 | 145 | 170 | 7.4 | 5.31 |
| | 95 | 85 | 7.37 | 6.19 |
| 3 | 165 | 170 | 7.59 | 4.14 |
| 4 | 130 | 170 | 7.57 | 5.65 |
| 5 | 110 | 105 | 7.47 | 4.79 |
| 6 | 90 | 115 | 7.31 | 4.98 |
| 7 | 145 | 175 | 7.43 | 4.15 |
| 8 | 130 mm table | 170 | 7.47 | 4.31 |
| 9 | 130 mm table | 105 | 7.34 | 3.45 |
| 10 | 170 unstable | 100 | 7.45 | 4.0 |

Table Blood flow in g/min in different tissue during unilateral cervical sympathetic stimulation (mean \pm S.E. 10)

| Tissue | 15-25 | | 5 min | |
|----------------|-----------------|-----------------|-----------------|-----------------|
| | Stim. side | Contr. side | Stim. side | Contr. side |
| Cerebrum | 0.39 \pm 0.03 | 0.41 \pm 0.03 | 0.39 \pm 0.03 | 0.43 \pm 0.03 |
| Anterior part | 0.38 \pm 0.03 | 0.47 \pm 0.03 | 0.43 \pm 0.03 | 0.41 \pm 0.03 |
| Posterior part | 0.43 \pm 0.03 | 0.47 \pm 0.03 | 0.45 \pm 0.03 | 0.50 \pm 0.03 |
| Cerebellum | 0.45 \pm 0.03 | 0.42 \pm 0.03 | 0.47 \pm 0.03 | 0.48 \pm 0.03 |
| Choroid plexus | 1.1 \pm 0.58 | 1.78 \pm 0.68 | 1.86 \pm 0.53 | 2.07 \pm 0.57 |
| Optic nerve | 0.52 \pm 0.08 | 0.53 \pm 0.10 | 0.51 \pm 0.09 | 0.54 \pm 0.13 |

on stimulated side, and there was no difference between the first and second measurement. In the anterior part there was a difference of approximately 10% between the stimulated and non-stimulated side, which was significant at the 5% level at both occasions.

No significant effect of sympathetic stimulation on the blood flow could be seen in the cerebellum, the optic nerve or the retina. In the choroid plexus a small difference of $22.0 \pm 6.6\%$ ($P < 0.01$) between stimulated and non-stimulated sides was noticed during paired comparisons. After 5 min there was no significant difference.

In the choroid, blood flow was initially reduced on the stimulated side by $26.0 \pm 1.0\%$ and by $41.9 \pm 9.5\%$ 5 min later. The corresponding figures for the iris were $33.0 \pm 21.0\%$ and $41.0 \pm 18.0\%$. The ocular blood flow was reduced by $3.0 \pm 8.0\%$ on the stimulated side at the first injection. This difference was significant ($P < 0.05$). After 5 min the difference was $24.0 \pm 6.0\%$ ($P < 0.05$). There was a good agreement between the flow values calculated from data for $8.7 \mu\text{m}$ spheres and calculations using data for $15 \mu\text{m}$ spheres.

The blood flow values for the masseter muscle are presented in Table 4. In 8 of 10 the blood flow on the stimulated side was almost stopped during the first measurement and then increased

DISCUSSION

Histological studies by Naekken & Olsman (1967) on cats have shown that the anterior cerebral artery and cerebellum receive adrenergic innervation from the superior cervical ganglion both on the ipsilateral side and to some extent from the contralateral side. But Helstad et al (1978) stated that the functional importance of innervation to the contralateral hemisphere is not great. Lacombe et al (1977) demonstrated that CBF in non-stimulated rabbits did not differ from CBF on the non-stimulated side in rabbits exposed to unilateral stimulation. However, according to their study there might be some nervous cross-over in the vessels supplying the optic nerve. In the inner ear of the cat, part of the vascular innervation seems to originate in the contralateral stellate ganglion (Spoendlin 1969) but Dersert (1974) showed that the sympathetic innervation of the rabbit cochlea derives only from the ipsilateral superior cervical ganglion. As these latter functional studies showed no larger cross-over of the sympathetic nerves, rabbits were used in this study.

For blood flow measurements in small organs, such as the cochlea, it is necessary to use large amounts of spheres. This is possible when using small spheres, but if they are too small the spheres may pass through the tissue. Recent studies indi-

Table 3 Blood flow in whole tissue in different organs during unilateral sympathetic stimulation (mean \pm S.E. 10)

| Tissue | 15-25 | | 5 min | |
|-----------|------------------|------------------|------------------|-----------------|
| | Stim. side | Contr. side | Stim. side | Contr. side |
| Anterior | 5.66 \pm 1.76 | 6.32 \pm 1.14 | 3.96 \pm 1.14 | 5.50 \pm 0.93 |
| Posterior | 33.48 \pm 8.10 | 49.68 \pm 9.90 | 21.89 \pm 5.73 | 31.98 \pm 9.5 |
| Choroid | 660.70 \pm 88 | 848.73 \pm 85 | 334.13 \pm 54 | 639.20 \pm 85 |
| Cochlea | 2.5 \pm 0.38 | 3.33 \pm 0.41 | 2.9 \pm 0.35 | 3.81 \pm 0.44 |

Table 4 Blood flow in $\text{g min}^{-1} \text{g}^{-1}$ in the masseter muscle during unilateral cervical sympathetic stimulation

Measurements after 15–25 s (inj. I) and 5 min (inj. II)

| Exp no | Stimulated side | | Control side | |
|--------------------------------------|-----------------|---------|--------------|---------|
| | Inj. I | Inj. II | Inj. I | Inj. II |
| <i>Small spheres both injections</i> | | | | |
| 1 | 0.0081 | 0.79 | 0.26 | 0.15 |
| 2 | 0.0011 | 0.016 | 0.26 | 0.1 |
| 3 | 0.0097 | 0.10 | 1.04 | 0.48 |
| 4 | 0.11 | 0.086 | 0.5 | 0.13 |
| 5 | 0.0002 | 0.0039 | 0.49 | 0.30 |
| 6 | 0.0009 | 0.023 | 0.5 | 0.29 |
| 7 | 0.0005 | 0.0076 | 1.44 | 0.36 |
| <i>Large spheres first injection</i> | | | | |
| 8 | 0.15 | 0.078 | 0.60 | 0.11 |
| 9 | 0.001 | 0.027 | 1.08 | 0.39 |
| 10 | 0.004 | 0.009 | 0.39 | 0.14 |

cate that 8–10 μm spheres tend to pass through the iris and ciliary body (Stjernschantz Alm & Bill 1976) and that $8.2 \pm 0.8 \mu\text{m}$ spheres tend to pass through the brain (Hultcrantz 1979). In the present study no clear tendency of passage was seen with 9.0 or 8.7 μm spheres in any of the organs investigated.

The differences in mean arterial blood pressure in the animals were probably at least partly due to different ages and maturation but it is well known that cerebral blood flow is efficiently autoregulated within a wide range of blood pressures. In the eye of the rabbit the retina is well autoregulated but in other parts autoregulation is poor. The cochlea also has a rather constant flow at normal blood pressures (Hultcrantz et al 1977). Alm & Bill (1973) have shown that unilateral electrical stimulation of the cervical sympathetic chain has little or no effect on cerebral and retinal blood flow. Under the same conditions Hultcrantz & Angelborg (1977) measured a 25% reduction in cochlear blood flow. The microsphere method was used on cats in both these studies. Lacombe et al (1977) using the ethanol technique and the local thermoclearance technique in anesthetized rabbits reported reductions in flow of 12–29% during sympathetic stimulation, the effect varying from one place to another. Lassen (1974) reviewed the literature on sympathetic effects on cerebral vessels and concluded that stimulation probably gives a slight vasoconstriction and a

flow reduction of about 10% in normotension. Our results agree with this view. In hypertension a more pronounced vasoconstriction is observed during sympathetic stimulation (Bill & Linder 1976).

When microspheres are used the time measured equals the injection time plus a few seconds since the spheres disperse almost immediately out into the tissues. The microsphere technique used in the present experiments thus enabled registration of the transient flow change occurring 15–25 s after stimulation started. This instant of time interval seems the most interesting since prolonged stimulation of the sympathetics might result in a reduction of the response, an "autoregulatory escape". In the literature the maximal constrictor effects appears at 20–25 s (Dresel et al 1966) whereafter it decreases. In the present study a possible autoregulatory escape of this kind could only be seen in the masseter muscle (see Table 4). This tissue has a very rich sympathetic innervation causing vasoconstriction with an extreme reduction in blood flow when stimulated (Table 4) as seen also in previous studies (Alm & Bill 1973; Hultcrantz & Angelborg 1978).

It is somewhat surprising that the effect of sympathetic stimulation seemed to be more marked in the posterior part of the brain than in the anterior part (Table 2). The anterior part has been reported to have a richer adrenergic innervation than the posterior part (Owman et al 1974). The lack of effect of sympathetic stimulation on the blood flow in the retina and the optic nerve shows good agreement with previous reports (Alm & Bill 1973). Thus in normotension it would not appear probable that the sympathetics play a role in the regulation of the blood flow in the retina and the optic nerve. An important vasoconstrictive effect and protection against overperfusion has been observed in acute arterial hypertension (Bill, Linder & Linder 1977).

The role of the sympathetic nerves in the control of cochlear blood flow is not known. The observation of Borg (1977) that noise tends to cause vasoconstriction in the tail of the rat might also apply to the cochlear blood circulation. However, recent results by Hultcrantz (1979) showed no vasoconstrictive effect of noise on the cochlear blood flow. Further studies are planned to study whether the sympathetic nervous system plays a role in protection against overperfusion during acute hypertension as in the brain.

Thus no indication was found for an auto-

respiratory escape phenomenon in the brain the
of the cochlea of anesthetized rabbits.

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Transfer properties of the slowly adapting stretch receptor of the crayfish abdomen

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The slowly adapting stretch receptor in the abdomen of freshwater crayfish (*Astacus fluviatilis*) was investigated to determine its properties under dynamic conditions. An *in situ* preparation was used; the necessary dissection did not involve the receptor organ or its associated neurosomata. Sinusoidal variations in the angle of flexion in the joint to which the receptor organ was connected were generated by feed-back controlled stretcher. Nervous spikes recorded from the axons of the receptor neurons and information about angle of flexion in the joint obtained by position transducers, were fed into a computer. Fourier transforms were performed on both input and output data to determine the amplitude of the 0 and 1 harmonic together with the phase of the 1 harmonic. The receptor organ was investigated for linearity up to 1.5 degrees input amplitude and proved to be surprisingly linear within this range. In addition the transfer function of the receptor organ was determined by stimulating it with small-amplitude sinusoids with different frequencies. With steady flexion of 35-40° in the joint the gain of the receptor organ increased 5-6 times when the modulation frequency of the input signal was increased from 0.1 to 5 cycles/s. A maximum in gain was consistently found at about 5 cycles/s, with rapid fall towards 0 when the modulation frequency was increased further. A change in phase lead from positive (leading output) to negative with change in sign about 1 cycle/ was also found. These results resemble the results found by investigators of isolated preparations. A 'hold' property is probably part of the overall property of the receptor organ together with an element of Maxwell type. An element of the form $h(s) = ks^2$ with $k = 0.45$ is also part of the transfer function of the receptor organ although the physiological parallel to this element is uncertain.

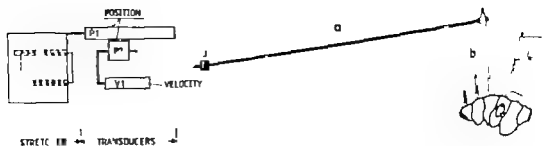
First described by Alexandrowicz (1951), the stretch receptor organ of the crustacean abdomen has been the subject of many investigations (for references see Alexandrowicz 1967) as well as physiological (for references see Terzuolo & Kato 1971). In crustaceans each abdominal segment is equipped with two pairs of receptors situated dorsally symmetrically near the midline. Each receptor pair consists of one rapidly adapting and one slowly adapting receptor. Only the slowly adapting receptor has been studied in the present investigation.

Any receptor organ may be regarded as a system which transforms one kind of information (input) into another form (output). If the system is linear it can be represented by a transfer function which is a particularly useful way of describing the transfer properties. The transfer function may be found by

the study of the response of the system to certain input signals. Sinusoids are widely used signals in such investigations, as well as white noise signals. The response of a linear system to a sinusoidal input will be sinusoidal while the response on nonlinear systems is normally not a sinusoid.

The transfer properties of the isolated receptor organ of the crayfish abdomen have been investigated earlier both under static and dynamic conditions (Terzuolo & Washizu 1962, Brown & Stein 1966). These authors found a linear relationship between the length of the receptor muscle and the firing frequency of the slowly adapting receptor under static stretches. However Njå & Walløe (1973) investigated the receptor *in situ* under static stretches and found a power-function relationship with exponent equal to 2 between input and output. Whether or not the dissection and preparation

A



B



C

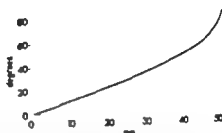


Fig. 1 (A) Experimental setup. To the right the crayfish abdomen showing the site of dissection of the nerve from the stretch receptor. To the left feedback controlled stretcher and transducers. P1 position transducer measuring position of stretcher. P2 position transducer (differential transducer) measuring position of the coil of the stretcher. V1 velocity transducer. P2 and V1 gives feedback to the controller. J marks ball joint coupling between stretcher and preparation. The position is measured by P1 and P2. (B) Geometrical representation of the setup. Origin corresponds to point J (see above). a, b, c and x_0 are measured in the setup. dx difference from zero position of point J measured by P1 and P2. α corresponding angle of flexion in the joint. (C) α as a function of dx. The actual values measured on a setup were $a=140.4$ mm, $b=49.9$ mm, $c=150.8$ mm, and $x_0=144.8$ mm. A linear relation between dx and α in the physiological range (0–42°) is seen.

methods used to obtain the isolated preparations explain the differences between these earlier investigations is uncertain. The non-uniformity of the receptor muscle bundle found both anatomically (Bodian & Bergman 1962) and physiologically (Wendler 1963) indicates, however, that any manipulation of the receptor muscle might well interfere with the transfer properties of the receptor organ.

We therefore decided to re-examine the transfer properties of the receptor organ under dynamic conditions using an *in situ* preparation.

Some of the results have been presented to a meeting of The Scandinavian Physiological Society (Sydnes 1979).

METHODS

Dissection and mounting of the preparation

The experiments were performed on freshwater crayfish *Astacus fluviatilis*. Removal of the thorax left an isolated abdomen which could be kept alive for hours. During the dissection the abdomen was kept in cold saline (5–10°C)

containing (mmol/l): NaCl 70.5, KCl 5.4, CaCl_2 13.5, MgCl_2 6, Tris 10, titrated to pH 7.4 with maleic acid. A hole was bored in each tergite in the dorsal midline and glass rods screwed into the holes. Dental cement was used to fasten the glass rods and fix all joints except the third in zero position as shown in Fig. 1. A hole was then drilled in the third tergite on the right side of the abdomen (see Figs 1 and 2) and the underlying connective tissue membranes were removed. Usually the nerve containing the afferent axon from the receptor could easily be found embedded in the loose connective tissue covering the large flexor muscle bundle. The nerve was dissected free over the greatest possible length and cut centrally. After dissection the preparation was moved to a bath containing oxygenated saline with thermostatically controlled temperature of 11°C and the glass rod of the fourth tergite was coupled to a stretcher. The central end of the cut nerve was drawn into a suction electrode and the signal fed to an amplifier.

The resulting experimental setup is shown in Fig. 1A. The stretcher was feedback controlled by signals from one position and one velocity transducer. The reference signal was generated by a Wavetek 184 signal generator. Information about the position in the joint was obtained from two position transducers, one of which measured large movement, the other small fluctuations in joint angle. The latter was also part of the feed-back loop controlling

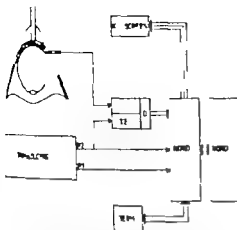


Fig. 2. Coupling between setup and computers (NORD-1 and NORD-10). Transducer symbols as in Fig. 1A. T1 and T2 are level-trigger parts of lab-box. D: digital part of lab-box.

the stretcher. Both transducers were calibrated and found to behave linearly. Using calibration data, every change in angle of flexion in the third abdominal segment could be obtained from the geometry of the system, as shown in Fig. 1A and 1B. The curve in Fig. 1C shows the angle of flexion as function of deviation in point J (Fig. 1A) from its rest position. All parameters from an actual preparation, however, showed that this curve changed very little as long as the changes of the parameters were reasonable and that the function was very nearly linear within the physiological range of the angle of flexion (0–42°).

Readout of the receptor

The experiments were performed in the following manner. The stretcher was stationary moved to preset position, resulting in steady flexion in the joint of 35–40°. This steady stretch was measured by transducer P1 (Fig. 1A) (2). After 15 s of adaptation, sinusoidal voltage starting at zero was sent to the reference input of the feedback controller of the stretcher. This resulted in sinusoidal output from transducer P2 (Fig. 1A), and hence sinusoidal variation in angle of flexion around the steady flexion in the joint. The traces *a* and *b* in Fig. 3A show the flexion from P1 together with the resulting spike train with its periodic variations.

Computer hardware

The preparation was coupled to the computer via one digital and two analog lines as shown in Fig. 2. The analog lines were read in conventional way by NORD-1 computer, and carried signals from the position transducers, thus enabling calculation of the angle of flexion in the joint at any wanted moment. The digital line carried information from level-discriminator giving trigger signals. However its preset level was passed in one direction. Our level discriminator had two separate inputs one of which received the spike train from the nerve. The signal from

transducer P2 was sent to the other input, thus generating a cycle-marking signal.

A FORTRAN written program was running in NORD-1 during the experiment, enabling on-line data processing and presentation as well as storage. On-line data were presented on an *y*-screen, and the action of the program controlled through a teletype (see Fig. 2).

Each triggering event arriving at the level-discriminated circuit was timed by clock-procedure. In our program, it was programmed to count millisecond intervals. This proved to give time resolution good enough for our purposes, since the firing frequency only seldom was higher than 20 imp/s.

Data processing

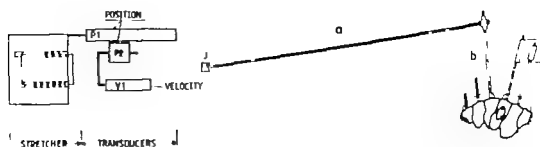
The data processing consisted of different modules which could be activated by commands from the terminal. Processing of data for presentation of instantaneous frequency was however done while nerve spikes were collected by the computer. The presentation of these data is shown in trace of Fig. 3A where the instantaneous frequency corresponding to each interspike interval of trace *b* is plotted showing nearly sinusoidal variation in the instantaneous frequency. The aim of the further data processing was to determine relevant parameters for this cyclic variation in the instantaneous frequency together with parameters characterizing the input sinusoidal.

To obtain good estimate for the parameters of the cyclic variation of the output, it was necessary especially at high modulation frequencies, to read the nerve impulse train over many input cycles. This was done by presetting the total reading time normally 20 s. This would result in nearly constant number of registered spikes, distributed over an increasing number of stimulation cycles when cycle time decreased. Traces in Fig. 3B shows the instantaneous frequency plotted one cycle upon the other by resetting of the plotting procedure each time the cycle mark arrived.

Each cycle was divided into number of bins, in these experiments 16 to make possible later analysis of our data by Fast Fourier Transform. Each spike was referred to one bin, and the mean of the interspike intervals calculated for each bin. Inversion of the value referred to a bin gave good estimate for the firing frequency corresponding to the midpoint of α . The set of values obtained in this way was then used as input to the fitting procedure.

At the end of the spike reading, the cycle length was known to the computer. This was then used for reading the input signals from transducer P1 and P2, shown in Fig. 1. 16 analog values were read from the cyclic signal of P2 together with the corresponding traces, followed by the reading of P1. From these data it was possible to calculate the angle of flexion at 16 moment in one cycle. The Fourier transform was used for finding the parameters of the two cyclic signals, giving information about the 0. and 1 harmonic together with the phase of the 1 harmonic. The results obtained by this procedure are shown in trace *d* in Fig. 3B where the continuous line shows the best sinusoidal fit to the 16 bin values, indicated by crosses. Trace *c* shows difference in phase found between input and output. Although it would be easy from mathematical point of view no higher harmonics were obtained

A



B



C

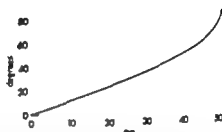


Fig. 1. (A) Experimental setup. To the right the crayfish abdomen showing the site of dissection of the nerve from the stretch receptor. To the left feedback controlled stretcher and transducers: P1 position transducer measuring position of stretcher; P2 position transducer (differential transducer) measuring position of the coil of the stretcher; V1 velocity transducer. P2 and V1 gives feedback to the controller. J marks ball joint coupling between stretcher and preparation. The position is measured by P1 and P2.

(B) Geometrical representation of the setup. Origin corresponds to point J (see above). a, b, c and d are measured in the setup. dx, difference from zero position of point J measured by P1 and P2. α corresponding angle of flexion in the joint.

(C) α as a function of dx. The actual values measured on a setup were: a = 150.4 mm, b = 49.9 mm, c = 150.8 mm, d = 144.8 mm. A linear relation between dx and α in the physiological range (0–42°) is seen.

methods used to obtain the isolated preparations explain the differences between these earlier investigations is uncertain. The non-uniformity of the receptor muscle bundle found both anatomically (Bodian & Bergman 1962) and physiologically (Wendler 1963) indicates however that any manipulation of the receptor muscle might well interfere with the transfer properties of the receptor organ.

We therefore decided to re-examine the transfer properties of the receptor organ under dynamic conditions using an *in situ* preparation.

Some of the results have been presented to a meeting of The Scandinavian Physiological Society (Sydnés 1979).

METHODS

Dissection and mounting of the preparation

The experiments were performed on freshwater crayfish *Astacus fluviatilis*. Removal of the thorax left an isolated abdomen which could be kept alive for hours. During the dissection the abdomen was kept in cold saline (5–10°C)

containing (mmol/l): NaCl 205, KCl 5.4, CaCl₂ 13.5, MgCl₂ 2.6, Tris 10, ultrafiltered to pH 7.4 with maleic acid. A hole was bored in each tergite in the dorsal midline and glass rods screwed into the holes. Dental cement was used to fasten the glass rods and fix all joints except the third joint as shown in Fig. 1. A hole was then drilled in the third tergite on the right side of the abdomen (see Fig. 1) and the underlying connective tissue membrane was removed. Usually the nerve containing the afferent axon from the receptor could easily be found embedded in the loose connective tissue covering the large flexor muscle bundle. This nerve was dissected free over the greatest possible length and cut centrally. After dissection the preparation was moved to a bath containing oxygenated saline with a thermostatically controlled temperature of 11°C and the glass rod of the fourth tergite was coupled to a stretcher. The central end of the cut nerve was drawn into a suction electrode and the signal fed to an amplifier.

The resulting experimental setup is shown in Fig. 1. The stretcher was feedback controlled by signals from the position and one velocity transducer. The reference signal was generated by a Wavetek 184 signal generator. Information about the position in the joint was obtained from two position transducers, one of which measured large movements, the other small fluctuations in joint angle. The latter was also part of the feed-back loop controller.

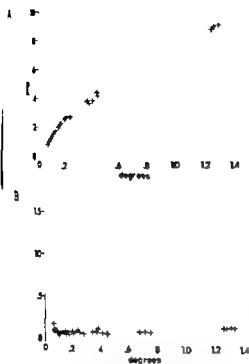


Fig. 4. Latency of the receptor response. (A) Output amplitude as function of input amplitude. Modulation frequency 1 cycle per second, steady stretch about 38° angle of knee. (B) Noise-to-signal ratio in the output as function of input amplitude. The relationship is calculated as phase difference per point divided by amplitude of first harmonic. Same experiment as in A.

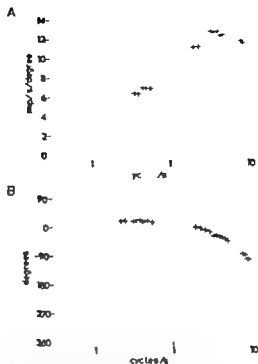


Fig. 5. Transfer properties of the receptor organ. (A) Gain as function of the modulation frequency in semi-logarithmic plot with linear vertical axis. Steady stretch $\sim 38^\circ$ input amplitude 0.2". (B) Phase difference between input and output as function of the modulation frequency. Positive phase difference corresponds to leading output.

ness, plotted in two semi-logarithmic diagrams. The upper part of the figure (A) shows the gain as a function of modulation frequency showing a four fold increase in amplification over the range 1 to 5 cycles per second. This large increase was a constant finding. The maximum was constantly found around 5 cycles per second. The experiments were always performed with modulations around a steady stretch of $35-40^\circ$ giving almost the same mean firing in every experiment. Therefore we have not made any correction or transformations of the frequency-axis as other investigators have done (Borsellino *et al.* 1965). One reason for always using a large steady stretch was the relatively low firing rate of the receptor after the initial adaptation. Lower steady stretch would result in an unacceptably low rate of firing, and silent period during the cycle, introducing greater uncertainty in the estimates of the parameters.

As one would expect taking phase locking

phenomena into account the gain falls rapidly towards zero when modulation frequency is increased towards the mean firing-frequency of the receptor. This behaviour also found by other investigators is usually interpreted as an effect of the holding properties of the coding process (Borsellino *et al.* 1965; Terzuolo & Korn 1971). To obtain information on transfer properties near this point it is necessary to prevent phase locking. This may be done either by applying very small amplitude inputs to the receptor or by adding noise to the input signal.

The lower part of Fig. 5 shows the phase difference as a function of the modulation frequency. The phase difference is defined as positive when the output leads to the input (as is shown in Fig. 3A). Fig. 3B shows a registration with negative phase difference (trace *f*). Regarded isolated the positive phase difference might be interpreted as an effect of velocity-sensitivity in the receptor. A constant el-

during the on-line computations. Neither were attempts made to fit sinusoids directly to the instantaneous frequency data.

RESULTS

Linear behaviour of the receptor

We decided first to find how linearly the receptor behaves under dynamic conditions. This was done by introducing sinusoids with constant frequency but with different amplitude to the receptor.

Fig. 4A shows the results from one such experiment with modulation frequency 1 cycles/s. The steady flexion in the joint was in this case about 38° and the mean firing frequency after adaptation for 15 s slightly above 10 impulses per second. A recording time of 20 s gave a total number of registered nerve spikes of about 210 or about 13 spikes per bin using 16 bins in the analysis of the spike train. The upper limit of the input amplitude (in this experiment about 1.3°) was partly determined by the movements of the nerve at the site of recording. Greater amplitudes gave instability in the recording and problems with the spike discrimination. At low values of the input amplitude the output became increasingly noise-dominated.

We also investigated alternative measures of linearity. Noise to-signal ratios based on both quadratic and absolute difference were examined together with higher harmonics. The noise to-signal relation expressed as (absolute difference per point)/(amplitude of first harmonic) is plotted as function of input amplitude in Fig. 4B. The high value of this parameter for small input amplitudes indicates noise domination. The slight increase in noise fraction when input amplitude was increased was also found in the other measures. When the lowest amplitude values were excluded, the higher harmonics ($n \geq 7$) all seemed to be linear functions of input amplitude. Expressed by means of amplitude of the first harmonic (A_1), a fairly constant relation seemed to be $A_2 = A_1/5$ and for n between 3 and 7 $A_n = A_1/70$.

The results indicate an approximately linear behaviour of the receptor under dynamic conditions. This was a common finding in our experiments also at other modulation frequencies. We also constantly found a slight decrease from strict proportionality when input amplitude was increased as illustrated in Fig. 4A, indicating a fall in the gain of the receptor for large input amplitudes.

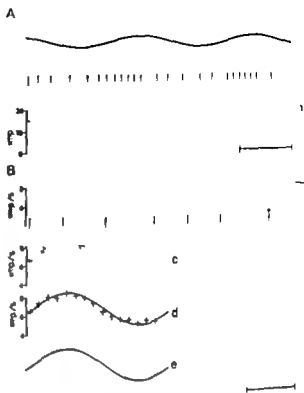


Fig. 3 (A) Registration from receptor with mean firing frequency of about 10 imp/s. Duration of modulation cycle 1 s. Trace a: Output from position transducer P_1 (or Fig. 1) as a function of time. Amplitude approximately 0.8° . Trace b: Corresponding spike train from the receptor, showing periodic variation in interspike intervals. Trace c: Corresponding instantaneous spike frequency. (B) Data obtained from a receptor with mean firing frequency of about 7 imp/s. Duration of modulation cycle 0.5 s. Input amplitude 11.5° . Trace a: Instantaneous spike frequencies corresponding to spike train in trace b. Trace b: Reconstructed spike train. Trace c: Instantaneous spike frequencies of all interspike interval in the experiment, plotted with resetting of the abscissa. Trace d: Crosses show the mean interspike interval in each of 16 bins. Full drawn line shows the best sinusoidal fit to the experimental points. Trace e: Best fitted sinusoidal from trace a (fully drawn line) and output from position transducer (P_1) (dotted line) plotted with arbitrary vertical scale to show the phase difference between the two. In this case the input to the receptor leads to the output from it, a situation referred to a negative phase difference here -9.6° .

Transfer properties

The transfer properties of the receptor organ were studied by using small sinusoids with different frequencies as input to the receptor. In about 15 expts. we tried to keep the amplitude approximately equal to $0.1-0.2^\circ$ corresponding to an amplitude in receptor muscle length of $1-10 \mu\text{m}$ (Njå & Walløe 1973). Fig. 5 shows the result of one such experi-

ized elements of the form $H(s) = 1/(s^2 + \gamma s + \beta)$ in the system, concluding from finding a flat section in the logarithmic plot that the damping factor γ is fairly large. The suggested value of both γ and β is 0.7 in their paper. This transfer element corresponds to a viscoelastic Maxwell-element which might be a property of the receptor muscle. The point to the extreme right in Fig. 6A and B does not show the expected effect of a Maxwell-element. The location of the maximum gain in Fig. 6A corresponds, with its maximum slightly below 10 cycles per second, to the findings of previous investigators. Both gain and phase data, however, suggest a lower damping factor than that found by Krogh and co-workers (1965) in the isolated receptor. This difference may be explained as by damage to the receptor muscle in the isolated preparation.

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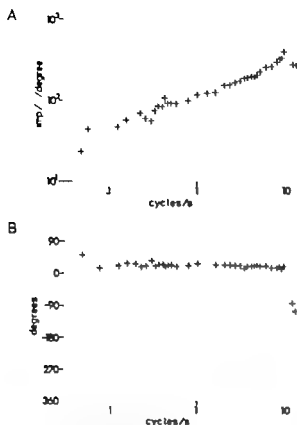


Fig. 6. Transfer properties after subtraction of zero order hold element with sampling time $T = 1/13.3$ s. (A) Gain data from Fig. 5A after division with the expected amplitude of a hold element. A double logarithmic plot shows nearly linear relation in this system. (B) Phase data from Fig. 5B after subtraction of expected effect of hold element.

ocuity sensitivity cannot however explain the fall in phase difference which is observed when the modulation frequency is increased. In our experiments the phase difference changed sign from positive to negative at slightly above 1 cycle per second.

Fig. 6 shows gain and phase difference respectively when the effect of the zero order hold element has been subtracted. The fairly linear increase of the gain function is obvious as in the horizontal course of the phase difference.

Finally one factor which influences the phase lag ought to be mentioned, namely the conduction time of the nerve spike from site of generation to site of recording. From previous investigations on the same preparation we have estimated the conduction time to about 5 ms. This would give a small negative phase difference increasing with increasing modulation frequency. The phase effect of this would be $\pi/20 = 9^\circ$ when modulation frequency is 10 cycles/s. Hence this effect may be disregarded.

DISCUSSION

The results show that the slowly adapting stretch receptor behaves quite linearly in the amplitude range from 0 to 1.5° when it is modulated around steady flexion in the interval 30 to 4° . For small steady flexion the linearity property is difficult to investigate by the present methods.

The other main result from the investigation is that the slope of the proportionality line between input amplitude and output amplitude (as shown in Fig. 4A) is about 6 times steeper for modulation frequencies near 5 cycles per second than for modulation frequencies near 0.1 cycles per second.

Most investigators agree that properties resembling a zero order hold element are important parts of the overall dynamic properties of receptor organ investigated by spike sampling procedure (Terzuolo & Knox 1971). This hold property may well be the combined effect of the encoder action of the receptor cell and the analyzing process in the computer. Every hold element consists of two distinguishable processes (Elgerd 1967): a sampling process which may here correspond to the spike generation process and the holding property corresponding to properties of the analyzing process. By dividing the total transfer function of the receptor organ by the transfer function of a zero order hold element one will obtain separate information about the transfer properties of the earlier stages of the signal processing in the receptor organ. The result of such a division is shown in Fig. 6. The data are those from Fig. 5. In the case plotted in Fig. 6 the sampling time T of the sample and hold element was set equal to $1/13.3$ s, a value obtained by root fitting to the data in Fig. 5. A maximum in the gain curve after subtraction of the hold element is recognized. This maximum however is found at higher modulation frequency than the maximum for the total system. This difference is not due to the fairly rough estimation of T since the maximum does not move when this value is changed.

The gain data of Fig. 6A are plotted in a double logarithmic coordinate system. The gain seems to increase linearly suggesting a simple power function relation between gain and modulation frequency. In fitting a power function of the form $y = k \cdot x^n$ to the points in the linear part of the logarithmic plot we found $n = 0.45$. This corresponds to a transfer element of the form $h(s) = s^{-0.45}$.

Other investigators (Borsellino et al. 1965) have

The pulmonary vasoconstrictor response to hypoxia The hypoxia-sensitive site studied with a volatile inhibitor

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BJERTNÆS L., HAUGE A. & TORGRIMSEN T. The pulmonary vasoconstrictor response to hypoxia. The hypoxia-sensitive site studied with a volatile inhibitor. *Acta Physiol Scand* 1980, 109: 447-46. Received 15 Dec. 1979. ISSN 0001-6772. Institute of Physiology, University of Oslo and Institute of Occupational Health, Ministry of Labour and Municipal Affairs, Oslo, Norway.

Recent investigations have revealed that a number of inhalation anaesthetics, including halothane, inhibit the pulmonary vasoconstrictor response to hypoxia without affecting other vasoconstrictor stimuli. Various injectable anaesthetics do not show this effect. This discrepancy could be due either to different pharmacological properties or to the different routes of administration. There is no general agreement on whether the response to hypoxia is elicited mainly by arterial hypoxia or by blood hypoxemia. Here within the lungs hypoxia acts. This work is an attempt to localize the hypoxia-sensitive site employing halothane. We have studied the reduction of standardized vasoconstrictor responses to hypoxia during administration of halothane via (1) the airways, (2) the pulmonary artery and (3) the pulmonary veins (backward perfusion). Our experimental model has been two pairs of series-perfused hyperventilated isolated rat lungs. An equimolar concentration of halothane most effectively inhibits the response when presented to the alveoli, less when presented to the arterial and least when presented to the venous segments of the pulmonary vasculature. We suggest that the response to hypoxia is inhibited by halothane at some extravascular site on the arterial side of the pulmonary vasculature, functionally closer to the alveoli than to the responding vessels. A model which combines all the data into an unifying concept has been presented.

Key words: Lung vasoconstriction, vascular resistance, microcirculation, respiration, halothane.

Impaired pulmonary gas exchange frequently develops during general anesthesia (Marshall et al 1974). This condition has been suggested in part to be due to inhibition of the pulmonary vasoconstrictor response to hypoxia (Bjertnæs 1978), a mechanism which acts to divert blood from poorly ventilated regions within the lungs (von Euler & Liljestrand 1946).

In isolated rat lungs Bjertnæs (1977) found that pulmonary vasoconstriction in response to hypoxia is reduced in a dose-dependent fashion by several commonly used inhalation anaesthetics including halothane. The vascular reactivity to other constrictor stimuli is, however, fully maintained. According to this author, no reduction of the vaso-

constrictor response to hypoxia occurs following administration of various intravenous anaesthetics to the perfusate. This difference in mode of action could be due either to different pharmacological properties of the two groups of anaesthetics or to the different routes by which they were administered. It is tempting to link the different effects of the two groups of anaesthetics to the question of where within the lungs hypoxia acts. This latter question is much debated (Fishman 1976). Most of the investigators in the field are of the opinion that the response is triggered from hypoxia-sens-

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BJERTNÆS, L., HAUGE, A. & TORGRIMSEN, T. The pulmonary vasoconstrictor response to hypoxia. The hypoxia-sensitive site studied with a volatile inhibitor. *Acta Physiol Scand* 1980, 109: 447-462. Received 15 Dec 1979. ISSN 0001-6777. Institute of Physiology, University of Oslo and Institute of Occupational Health, Ministry of Labour and Municipal Affairs, Oslo, Norway.

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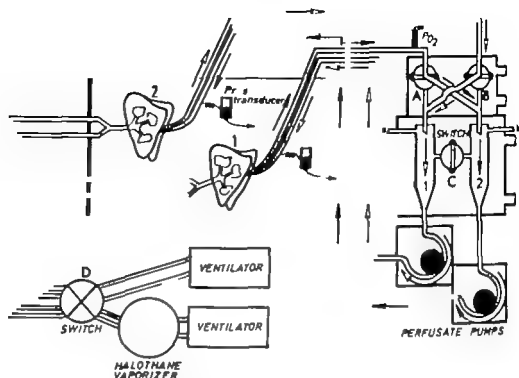


Fig. 1 Diagram of the experimental arrangement. The isolated rat lung preparations 1 and 2 (inside a thermostated perspex chamber) were perfused with blood by means of two pumps from reservoirs 1 and 2. Effluent blood could be drained back either to the same or to the reservoir of the other preparation (cross-perfusion) via the switches A and B. Switch C allowed perfusate overflow at the start of cross-perfusion. Pressure transducers were connected to the inflow and outflow tubings. P could be monitored in effluent blood from both preparations. A second cross-connection between preparation 1 and the pump allowed backward perfusion (not shown). Thin catheters were inserted via the side-bores a and b into the inflow tubings for blood sampling. Similar catheters were inserted into the effluent tubings. The preparations were ventilated with two ventilators, one connected to a halothane vaporizer. Switch D allowed the ventilators to be switched from the one to the other preparation.

tive site localized functionally closer to the alveoli than to the blood vessels (Bergofsky et al 1968, Hauge 1969, Hyman & Kadowitz 1973). Others, however, are in doubt about this alveolar theory (Fishman 1976). They are rather in favour of the view that the response is induced by a direct action of pulmonary arterial hypoxemia on vascular smooth muscle (Campbell et al 1967). If however the concept of alveolar hypoxia as being the main determinant is correct, the vasoconstrictor response to a constant hypoxic stimulus should expectedly be more readily damped by a given halothane concentration in the airways as compared to an equimolar concentration in influent blood under condition of low alveolar concentration.

The purpose of the present work has been to determine the main hypoxia-sensitive site in the lung with the use of a volatile inhibitor, halothane. The work can be looked upon as an attempt to test the "alveolar theory".

Instead of comparing the relative effect of alveo-

lar hypoxia and pulmonary arterial hypoxemia we decided to compare the reduction of a standardized vasoconstrictor response by halothane introduced (1) via the airways at low influent blood concentrations, and (2) via influent blood at low airway concentrations. In some of the preparations a sequence with backward perfusion was included in order to study the effect of pulmonary venous halothane.

METHODS

Rats of Wistar strain weighing between 240 and 260 g were anesthetized with diethyl ether. When unconscious, pentobarbital 3–4 mg/100 g body weight was injected intraperitoneally.

The animals were tracheotomized and continuous positive pressure ventilation was started keeping end-expiratory pressure at 1.5–2.0 cm H₂O. A mid-sternal thoracotomy was carried out and 0.5 ml of a solution containing 100 IU pure powdered heparin (Novo) was injected into the left ventricle. The heart-lung preparation was removed from the chest and cannulated as previously described (Bjertnæs 1977).

In every experiment, the lung preparations (no 1 and 2) were simultaneously perfused inside a thermostated (37°C) and humidified perspex chamber. The perfusate amount of 35-40 ml of isotonic heparinized (10 IU/ml) whole blood obtained by heart puncture of 3-5 sheep (Fig. 1) shows the experimental arrangement. Two roller pumps pumped the blood perfusate at constant flow from two reservoirs (1 and 2) into the vasculature of preparations 1 and 2 respectively. Until equal perfusate flow had been obtained through both preparations, clear blood from lung pair 1 was drained back (in reverse) and blood from lung pair 2 back to reservoir 1 and blood from lung pair 2 back to reservoir 2 (reciprocal perfusion). The blood reservoirs, tubes and the parts of the tubing lying outside the perspex chamber were also thermostated (37°C).

Perfusion in series ('cross-perfusion') was established by means of the venicles A and B. The effluent blood from preparation 1 was drained via switch A to reservoir 2 and from preparation 2 via switch B to reservoir 1. In this way the effluent blood from one lung preparation was pumped into the vasculature of the other. A third switch C was positioned between the reservoirs permitting per se flow at the start of cross-perfusion. This switch was closed as soon as both pumps had been adjusted to precisely the same flow. Two series of experiments were carried out in 6 of the experiments of series I as additional cross-perfusion as interposed in the perfusion circuit between preparation no 1 and the pump. This connection was designed in such a way that the same lung preparation could be perfused both forward via the pulmonary artery as well as backward, via the pulmonary vein. The left and right canulae used in these experiments, was fitted with two side-ports, one attached to an airtight tubing, the other to a pressure transducer. Mean pulmonary effluent blood pressure was continuously monitored from the pulmonary artery canula during forward perfusion and from the left arterial canula during backward perfusion by the use of Statham P 23 Db transducers connected to a Sanborn Series 2200 Recorder (Hewlett Packard). Since effluent blood pressure and outflow was kept constant, such preparation an increase in pulmonary vascular resistance as reflected as proportional increase in effluent blood pressure.

In some of the experiments pulmonary effluent P_{O_2} was measured in both preparations with oxygen electrodes (E 404, Radiometer Copenhagen). Each P electrode was connected through Physiological Gas Analyze Model 160 (Beckman) to the Sanborn Recorder.

Ventilation. The two lung pairs were independently ventilated at a frequency of 80 inflations per min. One of the preparations was ventilated with constant volume inflation by the use of Stirling 'Ideal' ventilation pump (C. F. Palmer Ltd. England). This respirator was connected to a halothane vaporizer (Drägerwerk, Lübeck, W. Germany). The other preparation was ventilated with constant peak pressure inflations by the use of Harvard Apparatus Respirator. Between the respirators and the lung preparation (Fig. 1) an interposed switch (D), which allowed both the respirator with the vaporizer and the without to be switched from the one preparation to the other.

Ventilation was started with 2-3 gentle hyperinflations

to peak pressure of 70 cm H_2O which was repeated before each experimental sequence. End-expiratory pressure was kept between 1.5 and 2.0 cm H_2O in both preparations by the use of water seals. The respirators were adjusted to give the same peak inflation pressure (8-10 cm H_2O) at the start of the experiment. The peak inflation pressure in the preparation which was ventilated with constant volume inflations was monitored continuously by means of a differential pressure transducer (Hewlett Packard Model 220) connected to the Sanborn recorder.

In both the experimental series the lung pairs were ventilated with 5% CO_2 in air (normoxic gas) for period of 8 min which were interrupted by 4 min periods of ventilation with gas containing 0% CO_2 , 5% CO_2 and 93% N_2 (hypoxic gas) in order to induce vasoconstriction.

Minute ventilation was measured with Collins dry spirometer.

Series 1 In these experiments both preparations were simultaneously ventilated with the hypoxic gas mixture. Since the preparations were perfused in series, the stimulus for vasoconstriction was combination of alveolar hypoxia and influent hypoxemia.

Series 2 In these experiments one of the preparations was ventilated with the hypoxic gas the other with the normoxic gas. Since the lung pairs were series-perfused the preparation ventilated with the hypoxic gas was perfused with blood with high Pa_{O_2} , the stimulus for vasoconstriction in these preparations was alveolar hypoxia only. The term influent blood used in this article is identical with pulmonary arterial blood during forward perfusion and pulmonary venous blood during backward perfusion. Correspondingly the term effluent blood means the blood coming from the lung vasculature irrespective of flow direction.

Determination of halothane in blood. Samples containing 200 μ l of blood were drawn with micropipette from effluent blood during halothane administration via the airways and from both influent and effluent blood during administration via the bloodstream. The blood samples were taken via small catheters, one inserted into the left atrial tubing the other into the pulmonary arterial tubing via side holes in the reservoirs (1 and 2) (Fig. 1). The blood specimen was diluted with 0.5 ml of water containing N-butanol as internal standard and sodium lauryl sulphate 1% as surfactant in 1 ml microvial properly sealed with airtight valves (Supelco Inc.). A volume of 1 μ l of this solution was injected into Perkin Elmer F11 gas-chromatograph equipped with flame ionization detector and 6 ft glass column packed with Chromosorb 102 and precolumn of teflochrome. Injection temperature was 150°C oven and detector were kept at 100 and 200°C respectively.

Standard solutions of halothane were prepared in water and peak height ratios were used for quantitative determinations. The peak height ratios were reduced according to the internal standard. In order to reduce possible concentration gradients, the samples were irradiated for 15 min in an ultrasonic bath before injection. At least 3 injections were carried out from each probe.

Determination of end-expiratory halothane concentrations. In 8 preparations of series I end-expiratory halothane concentrations were determined with Cen-

tronic mass spectrometer (Oth Century Electronics Ltd England) and recorded on a W+W recorder Model 314 (W+W Electronic Inc. Switzerland). Respiration gas was continuously sucked into the mass spectrometer at a flow rate of 4 ml/min through a cannula positioned in the trachea. The mass spectrometer was calibrated for concentrations of halothane ranging between 0 and 5% by means of the halothane vaporizer which again had been controlled by the use of a Riken gas indicator (Riken Kikai Instruments Co. Japan). In addition concentrations of halothane between 0 and 5% from the vaporizer were equilibrated at an air/halothane flow of 1.5 l/min with thermostated (37°C) water in a 100 ml impinger bottle with a glass frit (Bistabfl NS 79/3 W. Germany). Equilibrium was judged by the occurrence of equal halothane concentrations in the gas to and from the bottle. The halothane water concentration corresponding to 1% halothane in air was 5.98 mg/100 ml. Based on partition coefficients between blood and gas and water and gas of 2.36 v/v % and 0.77 v/v % (Lauenstein et al. 1979) respectively the corresponding blood concentration should be 18.35 mg/100 ml. The theoretical blood concentration corresponding to a gas concentration of 1% is 18.31 mg/100 ml as calculated on the basis of the molecular weight of halothane being 197.

Determination of pulmonary shunt flow. In 6 of the preparations of series 1 the pulmonary shunt fraction Q_v/Q_t was determined using the formula

$$Q_v/Q_t = \frac{C_c \text{ Hal} - C_a \text{ Hal}}{C_c \text{ Hal} - C_v \text{ Hal}}$$

The end-capillary halothane concentration $C_c \text{ Hal}$ was calculated on the basis of the end-expiratory concentration measured with the mass spectrometer (1% = 18.31 mg/100 ml). $C_a \text{ Hal}$ and $C_v \text{ Hal}$ are the halothane concentrations in pulmonary effluent and influent blood respectively. Q_v/Q_t was calculated following administration of halothane both via the airways and via blood.

Statistics. Unless another method is indicated Wilcoxon two-sample test (two-tailed) was used. The statistical evaluations were carried out by means of a computer programme for statistical routines on Nord 10 (Norsk Data A/S).

RESULTS

After having established series perfusion ("cross-perfusion") of two lung pairs standardized pulmonary vasoconstrictor responses to ventilation hypoxia ($F_v = 0.07$) were elicited as previously described (Hauge 1968; Bjertnæs 1977). Since flow was kept constant in each preparation changes in pulmonary arterial pressure directly reflected changes in vascular resistance. A reversible rise in pulmonary arterial pressure due to hypoxia is traditionally called a pressor response. Before any pharmacological interference two control responses of equal or slightly increasing magnitude were elicited. Halothane was administered from the

point of termination of the last of the control responses to the cessation of the next period of hypoxia.

Median minute ventilation in 3 lung pairs was 40 ml (range 78–84 ml) at peak inflation—and end-expiratory pressures of 10 and 2 cm H₂O respectively.

Series 1 Effects of halothane on the response to combined alveolar and influent blood hypoxia

Fig. 2 demonstrates typical pressor responses to combined alveolar and influent blood hypoxia and the concomitant reductions in pulmonary effluent blood P. In one pair of isolated lungs (Exp. 8 prep. 1) the upper panel shows two control responses followed by a test response which was reduced during administration of halothane via the pulmonary artery. In the next sequence (lower panel) the anesthetic was administered via the airways. On this occasion the response almost disappeared. Fig. 3 demonstrates from two typical experiments the relationship between percentage reduction of the pulmonary pressor response to hypoxia and the corresponding blood concentrations of halothane following both routes of administration. Open circles symbolize response reduction to halothane administered via the airways plotted against the resulting venous effluent blood concentrations. The latter values normally reflect the level of alveolar concentrations. Closed circles indicate response reduction during pulmonary arterial administration plotted against the concentrations in influent blood. Effluent blood concentrations are given in the parenthesis. Continuous lines combine results obtained in the same lungs. Ideal experimental conditions as regards the efficiency of alveolar ventilation are nearly obtained in the preparation presented to the left (Fig. 3). In this preparation (Exp. 3 prep. 1) the response was completely abolished during halothane administration via the airways giving an effluent blood concentration of 72 mg/100 ml. The influent blood concentration on this occasion was only 1.9 mg/100 ml due to effective elimination of halothane via the other lung pair. In comparison halothane in a concentration of 18 mg/100 ml administered via the pulmonary artery resulted in a 15% reduction of the response only. In this case effluent blood halothane concentration was as low as 2.3 mg/100 ml reflecting a very efficient "dehalothanization" of blood.

In several preparations however considerably

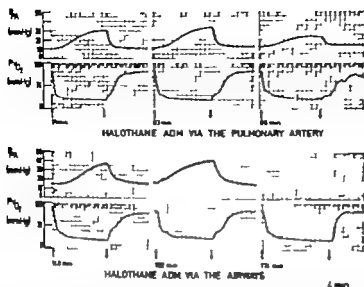


Fig. 2. Pulmonary pressure responses to hypoxia in one pair of isolated rat lungs before and during administration of halothane via the pulmonary artery (upper panel) and via the airways (lower panel). Concurrent reductions in effluent blood P are shown below the pressure tracings. Arrows indicate start and termination of hypoxic periods. Time from start of perfusion is given.

higher effluent blood concentrations were seen during administration via the pulmonary artery although the pressor response was fairly well maintained. For instance, in the preparation presented to the right in Fig. 3 which is identical with that shown in Fig. 2 the effluent blood concentration of halothane was 88 mg/100 ml. The corresponding value in effluent blood was 20 mg/100 ml. The reason for this relatively high effluent blood concentration is outlined below. In spite of the high halothane concentration in effluent blood however the response was not reduced by more than 50%. In contrast, airway administration resulting in a concentration of 77 mg/100 ml in effluent blood, completely prevented the response (Fig. 4 in similar plot, including all the expts of this type). It is evident from this diagram that arterial (closed circles) is less efficient than alveolar administration. Table 1 gives a complete survey of the results obtained in 33 of a total number of 4 lung pairs. Results from the remainder of the preparations were discarded due to spontaneously falling control responses or the absence of any response at all. Perfusate flow, baseline inflow pressure (P_i), the mean of two control responses (ΔP mean) and the magnitude of the response during halothane administration (ΔP) is tabulated, as well as the percentual re-

sponse reduction and the blood concentrations of halothane.

Administration of halothane via the airways resulting in a median effluent blood concentration of 33 mg/100 ml (range 10–88 mg/100 ml) reduced the response with a median of 100% (range 71–100%). In comparison, a median reduction of 50% (range 0–79%) was obtained during administration of the anesthetic via the pulmonary artery giving median influent and effluent blood concentrations of 36 mg/100 ml (range 18–88 mg/100 ml) and 1 mg/100 ml (range –31 mg/100 ml) respectively. The difference between the concentrations of halothane is primarily a measure of alveolar ventilation in these preparations.

Median perfusate P_p in 8 of the expts. was 120 mmHg (range 110–128 mmHg) during ventilation with the normoxic gas and 55 mmHg (range 15–30 mmHg) during ventilation with the hypoxic gas.

In 6 expts. the effect of halothane administered in the pulmonary veins was studied during a period of backward perfusion preceding administrations via the airways and via the pulmonary artery. Table 2 lists the relevant data from this test sequence. The preparations can be identified by their individual expt and prep. nos. given in Table 1. Fig. 5 shows tracings of the pulmonary influent blood pressure

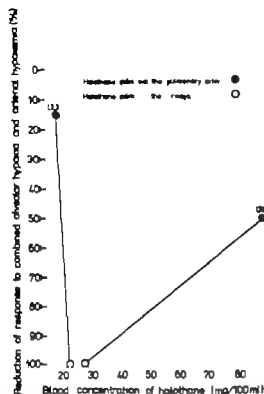


Fig 3 Reduction of pressor responses to hypoxia of halothane administered via the pulmonary artery and via the airways plotted against concentrations of the anesthetic in influent and effluent blood respectively. Lines connect tests in the same rat lung preparation. Numbers in parenthesis indicate effluent blood concentrations during arterial administration. In one of the preparations the latter concentration was low, in the other high.

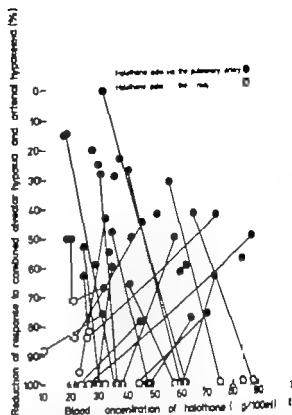


Fig 4 Reduction of pressor responses to hypoxia in the lungs of halothane administered via the pulmonary artery and via the airways plotted against the resulting concentrations in influent and effluent blood respectively. Lines combine tests within the same preparations.

and the effluent blood P from a typical preparation (Expt 20 prep 1). The responses demonstrated in the upper panel were obtained during backward perfusion. The two lower panels show tracings obtained during normal anterograde perfusion. During administration of halothane via the pulmonary veins resulting in concentrations of 37 mg/100 ml in influent blood and 17 mg/100 ml in effluent blood, the pressor response (upper panel) was reduced by 9%. Administration of the anesthetic via the pulmonary artery (middle panel) at concentrations in influent and effluent blood of 33 and 10 mg/100 ml respectively reduced the response with 43%. Finally halothane was administered via the airways resulting in a concentration of 23.5 mg/100 ml in effluent blood which reduced the response with 93%.

Fig 6 presents the relationship between reduction of response and blood concentration of halothane following administration of anesthetic via all the three routes. Reductions caused by venous (semiclosed circles) and arterial (closed circles) ad-

ministration respectively are plotted against the halothane concentrations in pulmonary influent blood. Simultaneously determined concentrations in effluent blood are shown in the parenthesis. Reductions caused by airway administration (open circles) are plotted against the resulting concentrations in effluent blood. In each lung preparation three levels of response reduction was found depending on whether halothane was given via the veins, the artery or the airways. Halothane administered via the pulmonary veins reduced the response with a median of 23.5% (range 8.3–62.4%). The median influent and effluent blood concentrations at this stage were 47 mg/100 ml (range 37–81 mg/100 ml) and 15.5 mg/100 ml (range 6–22 mg/100 ml) respectively. In comparison a median reduction of 38.5% (range 4.7–79.7%) was observed during pulmonary arterial administration resulting in median influent and effluent blood concentrations of 39 mg/100 ml (range 3.7–75 mg/100 ml) and 11.5 mg/100 ml (range 10–18 mg/100 ml), respectively. Airway administration reduced the re-

gave a median of 90.4% (range 71–100%) the median effluent blood concentration on this occasion being 3.5 mg/100 ml (range 1.0–39 mg/100 ml).

The damping effect of halothane following all the test routes of administration, could now be compared by using the ratio between reduction of response and blood concentration of anesthetic ("damping ratio"). The results are given in Table 3. Evaluation of the results with the Wilcoxon paired test (two-sided) shows that the ratio between percentual reduction of pressor response and influent blood concentrations of halothane is significantly smaller when the anesthetic is administered via the pulmonary artery as compared to administration via the pulmonary artery ($P=0.03$). Furthermore, the latter ratio is significantly smaller than the ratio between the percentual reduction of response and effluent blood concentration resulting from airway administration of halothane ($P=0.03$).

The median ratio between percentual reduction of response and effluent blood concentration of halothane during pulmonary arterial administration (0.37%/mg/100 ml), is higher than the similar ratio obtained during airway administration. This difference could be due to a combined effect of halothane acting in the wall of the pulmonary arteries and near the alveoli. If, however, one considers a single hypoxia-sensitive site only an equimolar concentration of halothane most effectively inhibits the pulmonary pressor response to hypoxia if presented to the alveoli, less if presented to the pulmonary arterial segments and least if presented to the venous segments.

During administration of halothane via the blood, surprisingly high effluent blood concentrations were observed in some of the preparations. We therefore calculated the shunt flow fraction (Q_v/Q_t). Table 4 gives a survey of the 8 preparations examined. The individual lung pairs can be identified by their expt. no. and prep. no. as referred to in Table 1. Concentrations of halothane in pulmonary venous, effluent and end-capillary blood are tabulated. The latter values were calculated on the basis of end-expiratory halothane concentrations determined with a mass-spectrometer. Q_v/Q_t was calculated at an early stage of the experiment and then upon one or two times during test responses. There was a tendency for Q_v/Q_t to increase from the first to the second or third measurement. For instance, in expt. 14, prep. 1, Q_v/Q_t increased from 0.03

shortly after the start of perfusion to 0.74 during a test response during exposure to halothane introduced via the pulmonary artery. In one of the preparations (expt. 1, prep. 1) Q_v/Q_t was determined also during retrograde perfusion.

A certain loss of halothane from tubings and reservoirs might be expected. The reduction in halothane blood concentration due to such loss was 10.3% (mean) from the effluent side of the lung pair being exposed via the airways to the influent side of the lungs exposed via blood.

Series 2 Effects of halothane on the response to alveolar hypoxia

In 10 experiments the period of ventilation hypoxia was confined to one of the lung pairs only while the other preparation was ventilated with normoxic gas. The median influent P_{O_2} during ventilation hypoxia in 4 of the test lung preparations was 118 mmHg (range 114–128 mmHg) whereas the simultaneously determined effluent blood P was 35 mmHg (range 22–42 mmHg). In spite of high influent blood P_{O_2} ventilation hypoxia induced pressor responses which were on the average only 13% smaller than those obtained in series 1. Fig. 7 shows pulmonary arterial pressure tracings from one typical preparation (expt. 28, prep. 2). Halothane administered via the airways resulting in an effluent blood concentration of 28 mg/100 ml reduced the response with 100% (upper tracing). In contrast, a pulmonary arterial halothane concentration of 70 mg/100 ml, reduced the response with 27% only (lower tracings). The corresponding effluent blood concentration was 5.5 mg/100 ml.

When halothane had been administered both via the pulmonary artery and via the airways in one of the two series-perfused preparations, the same procedure was repeated with the other one. Table 5 lists the complete results. Halothane was administered via both routes in a total of 10 pair of lungs. In 15 preparations administration was limited to one route only. The median reduction of the pressor response was 62.5 (range 0–82%) during halothane administration via the pulmonary artery giving a median influent blood concentration of 53 mg/100 ml (range 11–84 mg/100 ml). The simultaneously determined median concentration in pulmonary effluent blood reflecting the efficiency of alveolar ventilation was 14 mg/100 ml (range 3–34 mg/100 ml). Following administration of halothane via the airways, median reduction of response was 100%

Table 1 Comparison of the effect of pulmonary arterial and airway administration of halothane on pulmonary pressor response to combined alveolar hypoxia and arterial hypoxemia

| Exp no | Lung no | Flow (ml/min) | Halothane administration via the pulmonary artery | | | | | Halothane administration via the airways | | Halothane administration via the airways | |
|--------|---------|---------------|---|---|--|-------------|--|--|---------------|--|--|
| | | | Base line P (mmHg) | Responses before halothane adm ΔP mean (mmHg) | Response during adm of halothane ΔP_{Pa} | % reduction | Blood concentration of halothane mg/100 ml | Pulm influent | Pulm effluent | Base line P (mmHg) | Response before halothane adm ΔP mean (mmHg) |
| 1 | 1 | 20 | 17 | — | 6 | 67 | 5.0 | 16.0 | 19 | 22 | |
| | 2 | 20 | 16 | 22 | 9 | 59 | 7.0 | 7.0 | | | |
| 2 | 1 | 20 | 11 | 14.5 | 8 | 45 | 46.0 | 8.0 | 10 | 31.5 | |
| | 1 | 20 | 16 | 18 | 9 | 50 | 19.2 | 10.0 | | | |
| 3 | 1 | 15 | 10 | 13 | 11 | 15 | 18.0 | 2.3 | 13 | 1.5 | |
| | 2 | 15 | 17 | 10 | 5 | 50 | 20.0 | —3 | 10 | 11 | |
| 4 | 1 | 16 | 11 | 1 | 8.5 | 79 | 36.0 | 4.1 | | | |
| | 1 | 14 | 9 | 13 | 10 | 3 | 38.0 | 8.0 | | | |
| 6 | 1 | 18 | 16 | 10 | 5 | 50 | 4.0 | 14.0 | | | |
| | 1 | 18 | 19 | 9 | 4 | 55.5 | 34.0 | 14.0 | | | |
| | | 18 | 12 | 12 | 5 | 58 | 84.0 | 20.0 | | | |
| 7 | 1 | 15 | 13 | 8.5 | 4 | 53 | 25.0 | 14.0 | | | |
| | 2 | | | | | | | | | | |
| 8 | 1 | 20 | 11 | 1 | 11.5 | 50 | 88.0 | 20.0 | | | |
| | 1 | 20 | | | | | | | | | |
| | 2 | 20 | 20 | —5 | 9 | 60 | 62.0 | 31.0 | | | |
| 9 | 1 | 15 | 1 | 1.5 | 10 | 70 | 28.0 | 7.0 | | | |
| | 1 | 15 | 14 | 21 | 5 | 76 | 20.0 | 17.0 | 15 | 16 | |
| | 1 | 15 | 19 | 1.5 | 5 | 60 | 35.0 | 4.0 | 13 | 12 | |
| 10 | 1 | 16 | 10 | 18.5 | 13 | 7 | 41.0 | 3.5 | 15 | 16 | |
| | 2 | 16 | 15 | 22 | 15 | 31 | 56.0 | 3.5 | 15 | 26.5 | |
| 11 | 1 | 16 | 18 | 26 | 15 | 42 | 65.1 | 19.2 | 16 | 20 | |
| 12 | 1 | 18 | 15 | 17 | 6 | 64 | 73.6 | 21.3 | 17 | 22 | |
| 13 | 1 | 18 | 14 | 23.5 | 8 | 66 | 41 | 18.2 | 12 | 17.5 | |
| | | 18 | 14 | 23 | 5 | 78 | 64.0 | 24.9 | 15 | 25 | |
| 14 | 1 | 16 | 1 | 18 | 13 | 28 | 31.0 | 23.0 | 17 | 27 | |
| 15 | 1 | 18 | 10 | 33 | 19 | 42 | 51.0 | 1.0 | | | |
| | 2 | 18 | 18 | 28 | 21 | 25 | 30 | 16.2 | | | |
| 16 | 1 | 10 | 10 | 19 | 9.5 | 50 | 58.0 | 1.0 | | | |
| | 1 | 10 | 4 | 23 | 1 | 48 | 35.0 | 10.0 | 4 | 14 | |
| 17 | 1 | 10 | 10 | 14 | 3 | 79 | 45.0 | 12.0 | 14 | 10 | |
| | | 10 | 6 | 10.5 | 9 | 14 | 19.0 | 3.0 | 8 | 10 | |
| 18 | 1 | 12 | 16 | 15 | 5 | 67 | 32.0 | 11.0 | 15 | 17 | |
| | 2 | 1 | 16 | 20 | 1 | 0 | 3.0 | 5.0 | 10 | 17 | |
| 19 | 1 | 16 | 12 | 17 | 4 | 76 | 3.0 | 11.0 | 15 | 17 | |
| 20 | 1 | 18 | 19 | 21 | 1 | 43 | 33.0 | 10.0 | 20 | 29 | |
| 1 | 1 | 13 | 11.5 | 19 | 11 | 42 | 75.0 | 18.0 | 15 | 5 | |

(range 88–100%) which was obtained with a median effluent blood concentration of 29 mg/100 ml (range 24–56 mg/100 ml). Fig. 8 illustrates the reduction in response during administration of halothane via the pulmonary artery (closed circles) and via the airways (open circles) plotted against the concentrations of anesthetic in pulmonary influent and effluent blood respectively. Test points from the same preparations are connected by continuous lines. Statistical evaluation of the results shows that the ratio between reduction of response and effluent blood concentration of halothane following administration via the airways (median 3.45%/mg/100 ml (range 1.79–4.17%/mg/100 ml) is significantly higher ($P < 0.001$) than the ratio between the reduction of response and the influent blood concentration of halothane (median 1.04%/mg/100 ml (range 0.002–2.21%/mg/100 ml)) observed during pulmonary arterial administration.

DISCUSSION

A striking result of the present experiments was that equimolar concentrations of halothane reduced the

airway

mean
7 min
airway
reduction
Palm effluent
blood concen-
tration of
halothane
(mg/100 ml)

| | |
|-----|------|
| III | 77 |
| 84 | 22 |
| 100 | 22 |
| 100 | 21 |
| 100 | 84 |
| 100 | 31 |
| 100 | 33 |
| 100 | 77 |
| 100 | 30 |
| 100 | 45 |
| 100 | 30 |
| 100 | 60 |
| 100 | 75 |
| 100 | 88.6 |
| 100 | 61.6 |
| 100 | 58.3 |
| 100 | 48.8 |
| 100 | 37.8 |
| 100 | 39.0 |
| 100 | 48.0 |
| 100 | 4.0 |
| 100 | 37.0 |
| 71 | 21.0 |
| 100 | 6.0 |
| 88 | 10.0 |
| 93 | 23.5 |
| 84 | 26.0 |

lung ventilation thereby allowing the effect to be studied within the same preparation during administration via different routes. (3) Halothane is dissolved in blood in proportion to its blood/gas partition coefficient. End-capillary partial pressure of the anesthetic is equal to the alveolar partial pressure (Nunn 1969) in lungs with no or very small shunt flow fractions.

During blood administration of the anesthetic attempts were made to keep the effluent blood- and consequently also the alveolar concentration as low as possible by hyperventilation of the lungs. In this way the effect of halothane on the response could be confined to a vascular segment positioned upstream to the alveoli, i.e. an arterial segment during forward perfusion and a venous segment during backward perfusion. Likewise a low influent blood concentration was aimed at during airway administration.

The result of the two series of expts. will be discussed together. Although combined hypoxic stimulation (arterial+alveolar) gave somewhat larger responses, probably due to the lower perfusable P_{O_2} , no systematic differences were observed concerning the effects of halothane.

A close examination of Tables 1 and 5 reveals that during pulmonary arterial administration of halothane the low effluent blood concentration aimed at was obtained in about one fourth of the preparations (both preparations in expts. 3 and 10 preparation 1 in expts. 4, 3 and 25 and preparation

in expts. 17, 18 and 28). In most of these preparations the damping effect of halothane was small. In expts. 18 and 5 preparations and 1 no response reduction occurred at all in spite of influent blood halothane concentrations of 37 and 30 mg/100 ml respectively. In all the above lung preparations, the high concentrations of halothane must have been almost completely limited to the arterial segment and the contribution to response reduction caused by halothane in other lung regions negligible.

During airway administration the response in most of the preparations disappeared already when the effluent blood halothane concentration was between 20 and 30 mg/100 ml. In some preparations however much higher effluent concentrations were induced, the purpose being to establish a high influent blood concentration in the other pair of series-perfused lungs.

Let us now focus on the preparations in which halothane had been administered successively via

response more effectively when presented to the airways than when presented to the blood. When administration via the blood is considered more effective inhibition was obtained when halothane was presented in equimolar concentrations to the arteries than when presented to the veins.

There are several advantages in making use of an inhalation anesthetic to study the location of the hypoxia-sensitive site within the lungs. (1) The inhibition of the pressor response to hypoxia is reversible and specific (Bjertnes 1977). (2) A volatile anesthetic can be administered and eliminated by

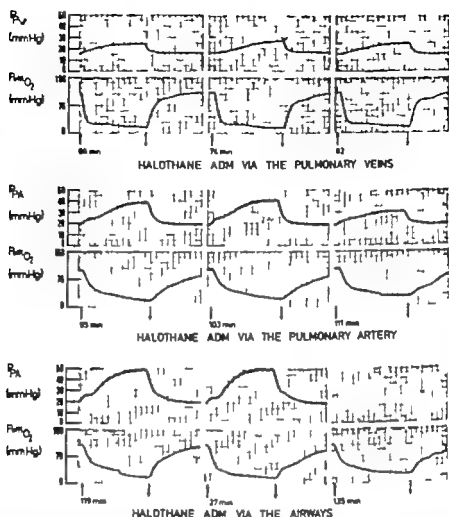


Fig. 5 Pulmonary pressor responses to hypoxia in one pair of (isolated rat lungs) before and during administration of halothane: in the pulmonary veins (upper panel), the pulmonary artery (middle panel) and the airways (lower panel). Cooccurrent reductions in effluent blood P_A are shown below the pressure tracings. Arrows indicate start and termination of hypoxic periods. Time from start of perfusion is given. Flow was constant (18 ml/min) regardless of flow direction.

all the three routes (Table 1 and 2). During venous administration the response was reduced with only 23.5% (median) in spite of an influent halothane blood concentration of 47 mg/100 ml (median). The corresponding effluent i.e. the pulmonary arterial concentration was then 15.5 mg/100 ml (median). When in contrast halothane was administered via the pulmonary artery, resulting in approximately 2.5 times the latter concentration, response reduction also increased 2.5 times. The alveolar concentrations on these occasions, as reflected by the effluent blood concentrations, were about the same (in medians 15.5 and 11.5 mg/100 ml during pulmonary venous and arterial administration respectively). These observations strongly indicate that when the alveolar concentration is constant, the reduction of

the pressor response to hypoxia is determined by the pulmonary arterial and not by the venous halothane concentration. A further implication is that the hypoxia sensitive region in the lungs is located functionally and probably also anatomically closer to the arterial than to the venous segment of the vasculature. This latter interpretation is consistent with the demonstration of mainly precapillary vasoconstriction in response to hypoxia in intact lungs (Kato & Staub 1966) and in isolated lungs from rats (Aarseth et al. 1980).

The damping effect of equimolar concentrations of halothane during administration via different routes could be compared directly by calculating the ratios between response reduction and the halothane concentrations in the different compar-

Table The effect on the pulmonary pressor response to combined blood/airway hypoxia of halothane administered in the pulmonary vein

| Exp. no. | Lung no. | Flow (ml/min) | Base line P_{PA} (mmHg) | Responses before halothane adm. | | Response during adm. of halothane | | Blood concentration of halothane mg/100 ml | |
|----------|----------|---------------|---------------------------|---------------------------------|------------------------|-----------------------------------|-------------|--|----------------|
| | | | | ΔP (mmHg) | mean ΔP (mmHg) | ΔP_{PA} | % reduction | Pulm. influent | Pulm. effluent |
| 8 | 1 | 10 | 9 | 1 | | 11 | 8.3 | 48.0 | 10.0 |
| 7 | 1 | 10 | 4 | 11 | | 7 | 36 | 37.0 | 19.0 |
| 9 | 1 | 12 | 13 | 13 | | 5 | 62 | 46.0 | 21.0 |
| 9 | 1 | 16 | 10 | 15 | | 9 | 40 | 38.0 | 6.0 |
| 3 | 1 | 18 | 15 | 11 | | 10 | 9 | 37.0 | 1.0 |
| 3 | 1 | 13 | 13 | 9 | | 8 | 11 | 81.0 | 22.0 |

test. These damping ratios obtained during pulmonary arterial and venous administration (Table 3), are only 42 and 16% respectively of that resulting from administration via the airways. The highest ratio was, however, found between response reduction and effluent blood concentration during administration of halothane via the pulmonary artery. It is a striking result that the difference between the latter ratio (5.20 %/mg/100 ml) and that resulting from airway administration (3.67 %/mg/100 ml) is identical to the ratio between response reduction and influent blood concentration obtained during arterial administration (11.33 %/mg/100 ml). Just as combined arterial and alveolar hypoxia is a more potent vasoconstrictor stimulus for alveolar hypoxia alone, so is a combined arterial and alveolar halothane administration a more efficient way to inhibit the response than alveolar administration alone. If we make the assumption that the actions are additive the reduction of the pressor response to hypoxia during arterial administration represents 29% of the total and the alveolar administration 71%. These findings are consistent with the earlier observations by Hauge (1969) that alveolar hypoxia and not arterial hypoxia is the main denominator for the pulmonary pressor response to hypoxia. He found that the types of stimuli interact in a way depending upon the relative intensity of ventilation and perfusion.

In a recent study also carried out in series-perfused rat lungs Bjertness & Mundal (1980) showed that the inhalation anaesthetic enflurane during administration via the airways reduced the pressor response to hypoxia in a fashion depending on

the end-expiratory enflurane concentration (determined by mass-spectrometry).

Since in the present study the ventilation/perfusion relationship was consistently high (4 to 8) it could be argued that more halothane is delivered by the airways than by blood in spite of the fact that

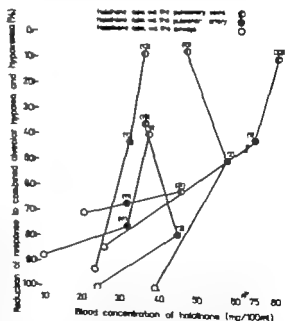


Fig. 6 Reduction of the pressor response to hypoxia of halothane administered via the pulmonary veins and artery plotted against effluent blood concentrations and via the airways plotted against effluent blood concentrations of the anaesthetic. Lines connect tests within the same isolated rat lung preparation. Numbers in parentheses indicate effluent blood concentrations during administration via blood.

Table 3 Median ratios between reduction of response to hypoxia (%) and blood concentration of halothane (mg/100 ml) during administration via three different routes

| Pulmonary veins | Pulmonary artery | Airways |
|---------------------------------------|---------------------------------------|---------------------------------------|
| 0.60 %/mg/100 ml (range 0.14–1.35) | 1.53 %/mg/100 ml (range 0.56–2.38) | 3.67 %/mg/100 ml (range 1.56–8.80) |

During airway administration the ratio was calculated on the basis of the effluent blood concentration whereas inflow blood concentrations were used for the other ratios.

the latter concentration in most cases was higher. This could then possibly explain the route-dependent difference in damping effect. If, however, the amount of halothane and not the partial pressure (proportional to the concentration) is the critical factor, the largest response reductions should be expected to occur in the preparations with the highest blood flow, and particularly in those with a combination of high inflow concentration and high flow. Such a combination was present in both preparations in expts 1, 7 and 8. The reduction of the pressor response observed in these preparations was, however, not larger than that seen in prepara-

tions with much lower blood flows. Ventilation was about the same in all preparations. In a study concerning the ability of alveolar hypoxia versus arterial or venous hypoxemia to elicit pulmonary vasoconstriction, Bergofsky et al. (1968) found that the response was mainly determined by alveolar P_{O_2} , irrespective of whether the ventilation/perfusion relationship had been 1.5 or 5. The response obtained during pulmonary venous or postcapillary hypoxemia was negligible.

The high effluent blood concentration of halothane observed in some of the preparations during administration via blood was found to

Table 4 Calculated fraction of right-to-left shunt (Q/Q_1) in isolated perfused rat lungs

| Exp. no. | Prep. no. | End-expir. halothane conc. (%) | Halothane blood concentration (mg/100 ml) | | | Q/Q_1 |
|----------|-----------|--------------------------------|---|--------------------|--------------------|---------|
| | | | End-capillary* | Pulmonary influent | Pulmonary effluent | |
| 11 | 1 | 4.90 | 89.77 | 3.70 | 88.60 | 0.02 |
| 11 | 1 | 0.70 | 3.66 | 65.10 | 19.70 | 0.25 |
| 12 | 1 | 4.40 | 80.56 | 21.30 | 79.90 | 0.01 |
| 12 | 1 | 0.25 | 4.60 | 60.5 | 17.80 | 0.4 |
| 13 | 1 | 3.80 | 69.38 | 4.90 | 58.30 | 0.25 |
| 13 | 1 | 0.70 | 3.66 | 41.70 | 18.20 | 0.39 |
| 13 | 2 | 5.00 | 91.55 | 1.10 | 87.30 | 0.07* |
| 13 | | 0.35 | 6.41 | 64.00 | 24.90 | 0.3 |
| 14 | 1 | 5.00 | 91.55 | 4.00 | 89.80 | 0.03 |
| 14 | 1 | 0.39 | 7.14 | 74.90 | 77.80 | 0.30 |
| 14 | 1 | 0.10 | 1.83 | 31.00 | 23.00 | 0.74 |
| 15 | 1 | 5.20 | 95.21 | 19.70 | 84.80 | 0.14* |
| 15 | 1 | 0.40 | 7.3 | 87.40 | 19.00 | 0.15 |
| 15 | 1 | 0.30 | 5.40 | 51.00 | 1.00 | 0.34 |
| 15 | | 5.30 | 97.04 | 19.00 | 97.80 | 0.05 |
| 15 | | 0.30 | 5.50 | 85.00 | 19.20 | 0.17 |
| 15 | 2 | 0.15 | 75 | 30.70 | 16.20 | 0.49 |
| 1 | 1 | 5.00 | 91.55 | 11.00 | 87.30 | 0.07* |
| 1 | 1 | 0.16 | 2.93 | 81.00 | 22.10 | 0.25 |
| 1 | 1 | 0.16 | 2.93 | 74.80 | 18.30 | 0.21 |

Calculated on the basis of measured end-expiratory halothane concentrations. Results obtained during airway administration of halothane just after start of perfusion. The other results obtained during influent blood administration of the anesthetic. Result obtained during retrograde perfusion.

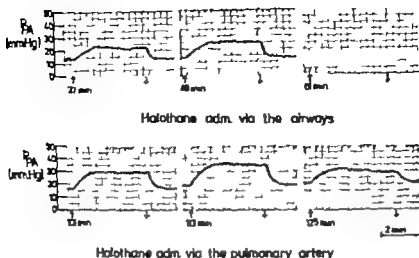


Fig. 7. Pulmonary pressure responses to alveolar hypoxia in one part of isolated rat lungs before and during administration of halothane in the airways and in the pulmonary artery. Arrows indicate start and termination of ventilation hypoxia. Time from start of perfusion is given as well. In contrast to the situations illustrated in Figs. 2 and 3 influent blood P_{50} was kept high (above 11.4 mmHg).

ated by a high calculated shunt flow fraction which tended to increase during perfusion. Whether the calculated shunt developed because of some mild deterioration of the lungs or because of a during ventilation/perfusion relationship due to hypoxia-induced vasoconstriction *per se* is not known. Histological examination of isolated rat lungs prepared ventilation and perfused for 1 h showed scattered regional differences in lung aeration, some regions being atelectatic others being hyperinflated (Bjertness et al. 1980). The calculated shunt flow could also be caused by uneven limitation of vasoconstriction during hypoxia resulting in decreased perfusion in some regions and overperfusion and high vascular pressures leading to edema development in others (Haltgren & Laver 1968). Whether the shunt-like effect as observed is due to true shunting of blood or to uncoupling of ventilation and perfusion is not known. Since the shunt was examined with a tracer with a very low partial pressure the shunt as observed is probably overestimated, most of it being due to ventilation/perfusion mismatching.

In the preparations with ventilation/perfusion ab-

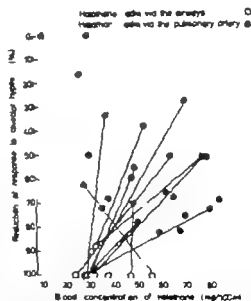


Fig. 8. Reduction of pressure responses to alveolar hypoxia (no influent blood hypoxemia) in rat lungs of halothane administered in the pulmonary artery and in the airways, plotted against the resulting concentrations in influent and effluent blood, respectively. Lines connect tests within the same preparations.

Table 5 Comparison of the effect of pulmonary arterial and airway administration of halothane on pulmonary pressor response to alveolar hypoxia

| Exp. no. | Lung no. | Flow (ml/min) | Halothane administration via the pulmonary artery | | | | | Halothane administration via the airways | | |
|----------|----------|---------------|---|---|--|-------------|--|--|---|-----|
| | | | Base line P (mmHg) | Responses before halothane adm. ΔP_{mean} (mmHg) | Response during adm. of halothane ΔP_{Pa} | % reduction | Blood concentration of halothane mg/100 ml | Base line P (mmHg) | Responses before halothane adm. ΔP_{mean} (mmHg) | |
| 22 | 1 | 15 | 70 | 17 | 3 | 75 | 70.0 | 8.0 | | |
| 23 | 1 | 15 | 15 | 1 | 6 | 50 | 30.0 | 4.0 | | |
| 24 | 1 | 10 | 77 | 15 | 5 | 67 | 38.0 | 14.0 | | |
| 24 | 2 | 10 | 2 | 18.5 | 5 | 77 | 35.0 | 14.0 | | |
| 25 | 1 | 14 | 14 | 11 | 11 | 0 | 30.0 | 7.0 | | |
| 25 | 1 | 14 | 18 | 11 | 3 | 82 | 59.0 | 14.0 | | |
| 25 | 2 | 14 | 70 | — | 9 | 59 | 48.0 | 1.0 | | |
| 26 | 1 | 1 | 19 | 18 | 5 | 77 | 80.0 | 14.0 | 4 | 3 |
| 26 | | 12 | | | | | | | 70 | 3 |
| 27 | 1 | 10 | 14 | 8 | 3 | 62.5 | 28.0 | 15.0 | 14 | 7.5 |
| 27 | 2 | 10 | 17 | 10 | 3 | 70 | 48.0 | 7.0 | 10 | 10 |
| 28 | 1 | 14 | 1 | 22 | 5 | 77 | 50.0 | 16.0 | 11 | 11 |
| 28 | 2 | 14 | 16 | 15 | 1 | 77 | 70.0 | 5.5 | 14 | 10 |
| 28 | | 14 | 18 | 14 | 7 | 50 | 79.0 | 7.0 | | |
| 28 | 2 | 14 | 4 | 17 | 19 | 0 | 11.0 | 3.0 | | |
| 29 | 1 | 1 | 1 | 9 | 4 | 55 | 49.0 | 9.0 | 15 | 10 |
| 30 | 1 | 14 | 18 | 20.5 | 10.5 | 50 | 78.0 | 4.0 | 1 | 10 |
| 30 | 1 | 14 | 25 | 77 | 5 | 81 | 68.0 | 34.0 | | |
| 30 | | 14 | 18 | 1 | 6 | 50 | 64.0 | 31.0 | 16 | 11 |
| 30 | | 14 | 30 | 1 | 4 | 67 | 65.0 | 4.5 | | |
| 30 | 1 | 14 | 25 | 25 | 1 | 16 | 26.0 | 18.0 | | |
| 31 | 1 | 14 | 14 | 16 | 10 | 37.5 | 43.0 | 15.0 | 11 | 9 |
| 31 | 1 | 14 | 70 | 14 | 4.5 | 68 | 84.0 | 8.0 | | |
| 31 | 2 | 14 | 19 | 15 | 10 | 13 | 37.0 | 16.0 | 18 | 9 |
| 31 | 2 | 14 | 24 | 13 | 4.5 | 65 | 62.0 | 8.0 | | |

normalities the end-expiratory halothane concentrations measured as concentrations in effluent blood might have been underestimated. This does not, however, appear to be the case. Mass spectrometrical measurements of end-expiratory halothane concentrations during airway administration in 8 preparations (Table 2) differed only slightly from those determined in effluent blood with gas chromatography.

Bearing in mind that small muscular arteries at the level of the terminal or respiratory bronchioles constrict in response to alveolar hypoxia (Kato & Staub 1966) it was not surprising to find that the response was least reduced when halothane was administered via the pulmonary veins. Thus the site from where the vasoconstrictor response to hypoxia is elicited must also be localized on the

arterial side of the pulmonary vasculature. Consequently blood should be expected to be more effectively "dehalothanized" before the hypoxia-sensitive region is reached during pulmonary venous than during arterial administration.

How can we explain the different susceptibility to halothane depending on whether the anesthetic is administered via the airways or via the pulmonary artery? Unless we know the profile of the halothane partial pressure curves from alveoli to the core of the responding vessels, no exact answer can be given. Such data are non-existent. An answer can, however, be approximated by extrapolating from information about oxygen diffusion from alveoli to small pulmonary arteries in lungs from cats ventilated with oxygen prior to rapid freezing (Staub 1961). In arteries with diameters below 200 μ and

| artery | |
|-----------------------------------|---|
| arteries during % of halothane | Pulm. effluent blood concentration halothane (mg/100 ml) |
| % reduction | |

| | |
|-----|------|
| 100 | 28.0 |
| 100 | 38.0 |
| 100 | 46.0 |
| 100 | 47.0 |
| 100 | 28.0 |
| 100 | 28.0 |
| 100 | 29.0 |
| 90 | 30.5 |
| 100 | 4.0 |
| 80 | 31.0 |
| 100 | 28.0 |

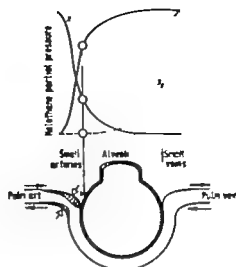


Fig. 9 The lower panel shows a schematic lung model with the proposed hypoxia-sensitive site () on the arterial side of the vasculature. The upper panel shows hypothetical halothane partial pressure curves along the pulmonary vasculature during administration via the airways (1) the pulmonary artery (2) and the pulmonary vein (3). The open circles symbolize the projection of the hypoxia-sensitive site on the halothane partial pressure curves, thereby indicating the assumed relation between the halothane partial pressures at this site during administration via the different routes. Continuous and broken arrows indicate flow direction whereas small arrows indicate the proposed vasoconstrictor site. Fig. 6 should be studied simultaneously.

outer zone of bright red blood was found whereas the vessel core was still magenta. Mainly on the basis of this observation Hauge (1969) proposed a flat oxygen tension gradient from alveolar wall to vessel lumen, and steep in blood interface. With improved technique Conham & Staub (1974) recently confirmed that 51% of pulmonary arteries with diameters between 475 and 200 μ taken from hyperventilated cat had an outer rim of blood whose oxygen saturation was 30% above that of the vessel core. The oxygen saturation of the other vessel cores corresponded to alveolar P_{O_2} , i.e. the P_{O_2} gradient from alveoli to the core of these vessels was almost quite flat.

In the present work, the response to hypoxia was more effectively damped during airway- than during blood administration of halothane. Such result is

hard to conceive in the presence of a flat halothane tension curve from the alveoli to the core of the responding vessels. With a flat, or almost flat curve the response should expectedly have been reduced by the same amount irrespective of airway- or blood administration. The marked difference related to the route of administration can however be explained by postulating a halothane gradient similar to that originally proposed for oxygen (Hauge 1969).

Fig. 9 shows hypothetical curves for the changes in halothane tension along the pulmonary vasculature during administration via the airways (1) the pulmonary artery (2) and the pulmonary vein (3). Below is a schematic model of the lung. The continuous and broken arrows indicate forward and backward perfusion respectively whereas the short arrows indicate the supposed site of vasoconstriction. Since the response to hypoxia is damped by halothane in dose-dependent fashion (Bjertnes

1977) the concentration of anesthetic in the hypoxia-sensitive site (circles in Fig. 9) should expectedly be least during pulmonary venous administration and sequentially higher during pulmonary arterial and airway administration respectively (Fig. 6). The model is compatible with all the data in the present work and supports the hypothesis that the region within the lungs sensitive to hypoxia is located extravascularly on the arterial side, functionally closer to the airways than to the responding vessels, as indicated (x) in Fig. 9.

At the beginning of the present study, L. B. was a research fellow of the Norwegian Council for Science and the Humanities. He is presently a research fellow of the University of Oslo. The work has been supported by grants from Norsk Medisinaldepot and from Instituttet i Helsevesen. We thank M. Karin Laugerud for skilled technical assistance.

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A simulation study of a neuron in a simple muscle control system

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LIESTØL, K., NJÅ, A. & WALLOE, L.: A simulation study of a neuron in a simple muscle control system. *Acta Physiol Scand* 1980, 109: 463-472. Received 20 Dec. 1979. ISSN 0001-6777. Institute of Physiology, University of Oslo, Norway.

The mechanism underlying signal transmission through the accessory neuron of the muscle receptor organ in the crayfish abdomen was examined experimentally and by computer simulation. The mathematical model of the accessory neuron and its synaptic input was based in part, on previous neuronal models studied by Stein (1965) and Rall (1964). The model predicted with considerable accuracy the behaviour of the experimental system in a number of situations. It was possible to determine in some detail the mechanisms underlying the frequency response characterized by a threshold and an approximately linearly increasing response for stimulus intensities above threshold. Furthermore, we were able to explain the observation that the accessory cell transforms a regular input from a stretch receptor into an irregular output train. The highly branched structure of the dendritic tree, combined with a firing threshold in the dendrites which prevents action potentials from entering large parts of the dendritic tree, seems essential in determining these properties.

Key words: Simulation, signal transmission, crayfish neuron.

The signal transmission through a neuron is determined by the interplay between several of its inherent properties. These may include the electrical characteristics of the nerve membrane, the geometry of the cell and the function of its synapses with other neurones. Since direct examination of these properties is often difficult, it is important to know to what extent the mechanisms underlying signal transmission can be deduced from the relationship between the input and output of the neuron. In the present study we have used extracellular stimulation and recording to examine one particular nerve cell, the large accessory neuron of the abdominal muscle receptor organ of the crayfish (Alexandrowicz 1951, 1967). We started by performing a set of experiments which served as a basis for the construction of a neuronal model. Preliminary analysis showed that a model more complex than those used by previous investigators in similar situations (Stein 1965, Walloe, Jansen & Nygaard 1969) was required. The result of computer simulation of the behaviour of this model were then compared with experimental observations.

Our results provide information about the roles of synaptic mechanisms and cell geometry in determining the signal transmitting properties of the accessory neuron.

METHODS

Experimental preparation

The dorsal muscles in the thorax and abdomen of several crustaceans possess large muscle receptor organs (Alexandrowicz 1951, 1952, 1967). Each of these sense organs consists of specialized muscle bundle and peripheral nerve cell body with highly branched dendrites that ramify the muscle. There are only four such receptors in each abdominal segment, two on each side. Each pair consists of a rapidly adapting and a slowly adapting stretch receptor, both of which are activated by abdominal flexion (Wiersma, Perran & Flörj 1953). The stretch receptor axons run ventrally across the surface of the body muscles and enter the abdominal ganglion of the next anterior segment. There are also 3 different axons making inhibitory synapses on the slowly adapting stretch receptor neuron (Alexandrowicz 1967, Jansen et al. 1971). The most powerful of these inhibitory axons is that of the large accessory cell, which is the object of this study. The slowly adapting receptor causes powerful reflex activation of the large

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A simulation study of a neuron in a simple muscle control system

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accessory neuron in the same and neighbouring segments (Eckert 1961). The accessory cells have been particularly useful in studies of the mechanism of synaptic inhibition (Kuffler & Eyzaguirre 1955). Inhibition in small nerve networks is investigated on principles of summation of excitatory and inhibitory impulses (Fenstad et al. 1976), lateral inhibition (Liestøl et al. 1976) and problems of phase locking (Perkel et al. 1964). In the present experiments we examined the correlation between the activity in an accessory cell and the activity in the stretch receptor that provided the reflex input. The experiments were performed on the isolated abdomen as previously described (Janßen, Njå & Walloe 1970; Njå & Walloe 1975). In short the superficial extensor muscles of each of the abdominal segments were exposed and a suction electrode placed on the muscle nerve for en passant recording of action potentials. The slowly adapting stretch receptor was either activated by flexion of the joint by stretching the receptor muscle with a hook, or the nerve was cut and its central end stimulated electrically. The preparation was kept in a bath of cold oxygenated physiological saline (12°C).

Intracellular recordings from abdominal ganglion cells were obtained on isolated preparations consisting of each pair of stretch receptors of the third abdominal segment and their nerves in continuity with part of the ventral nerve cord including the second and third abdominal ganglia. En passant recording and stimulation were done across a seal on each stretch receptor nerve (second root). The ganglia were pinned out ventral side up and decapsulated and conventional glass microelectrodes filled with 4 M potassium acetate (resistance 20–50 MΩ) were advanced through the cell body layer and into the neurone.

The model

Several mathematical models have been developed for the analyses of nerve mechanisms or for the evaluation of properties of specific neurons (Rall 1964; Stem 1965; Walloe et al. 1969; see also references in Holden 1976). However, certain properties of the accessory cells (to be described later) made it necessary to combine and extend these previous models.

Our model is defined by 6 assumptions. We will describe the model by stating these assumptions and by giving a motivation for each.

The model cell is divided into compartments, each representing a part of the neuron. Each compartment is regarded as an electrical unit, while both membrane properties and membrane potential may differ between compartments. The compartments are combined into a neuron by the assumption that current may pass between the compartments in a specified way.

Each compartment is based on a model originally formulated by Fatt & Katz (1953). All synaptic inputs are assumed to be excitatory (the model may easily be extended to include inhibitory input). The model may then be illustrated by means of an analogous electrical network. The model assumes that current may pass between membrane along three parallel channels. One channel represents the effect of the membrane capacitance (C). Each of the two other channels consists of an electromotive

force in series with a resistance. These two channels represent the effect of diffusion over the membrane under resting conditions and during excitatory influence. G_r (resting conductance) is constant while G_k is determined by the synaptic activity (to be described later). With no synaptic input ($G_k = 0$), the membrane potential (V) will be equal to the resting membrane potential (E_r). Synaptic activity will cause the membrane potential to approach the reversing potential for the excitatory synapse (E_k).

A mathematical formulation of these assumptions gives the differential equation

$$V_m = \frac{G_r}{C}(E_r - V) + \frac{G_k}{C}(E_k - V)$$

Following Rall (1964), we will introduce a new variable defined by

$$V = \frac{E_r - V_m}{E_r - E_k}$$

V may then be regarded as relative depolarisation, that is, V is equal to 0 under resting conditions and approaches 1 under strong excitatory input. The differential equation then takes the form

$$\dot{V} = -\frac{G_r}{C}V + \frac{G_k}{C}(1 - V)$$

Let g_{ij} be the conductance between the i th and j th compartment. We will then assume that the current between compartment i and j is equal to $g_{ij}(V_i - V_j)$. Furthermore, we will assume that the change in membrane potential induced by this current is proportional to the capacitance in the compartments. The conductance between compartments i and j will then result in a change in the membrane potential given by

$$\dot{V}_i = \frac{1}{C_i} \sum_j g_{ij}(V_j - V_i)$$

where $1/C_i$ are constant.

The neurons in the abdominal ganglia of the crayfish have long, highly branched dendrites (Wine & Hagiwara 1977). However, to obtain a practicable number of parameters in the model, we had to use a simple geometrical structure. Some exploratory trials showed that a two-branch structure was necessary to obtain satisfactory results when simulating situations with two inputs, e.g. controlled and spontaneous. We therefore selected the 16-compartment structure shown in Fig. 1. In most simulations, all segments were supposed to have equal membrane properties (with some exceptions, see below), i.e. all C , G_r and g_{ij} were equal for $i = 1, 2, \dots, 16$. This relatively simple choice of structure reduced the opportunities to study some properties of the accessory cells, especially summation of several inputs. However, several of the more important properties show little sensitivity to small changes in structure.

We may now state our first assumption.

(A1) The model cell is divided into compartments as shown in Fig. 1. Within each compartment the electrical properties are uniform. If generation of action potentials is suppressed, the variations in the membrane potential may

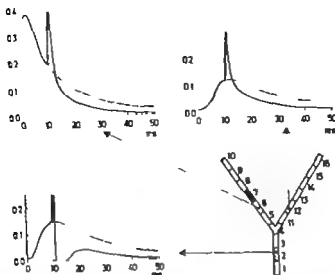


Fig. 1. Membrane potential as a function of time in three different segments of the models as a result of synaptic input in the black segment. Arbitrary vertical vs. Horizontal axis: time in ms. Dotted line: firing threshold. Broken lines show the response if the cell is not fired by the synaptic input. The fully drawn lines show the response when the cell fires.

is described by the following system of differential equations:

$$C \frac{dV}{dt} = \frac{G_p}{C} (1 - V) + \frac{k}{C} \sum_{j=1}^n \mu_j (V_j - V) = 1 \quad (16)$$

where

$\mu_j = \begin{cases} 1 & \text{if compartment } i \text{ and } j \text{ are connected} \\ 0 & \text{otherwise} \end{cases}$

and the other parameters have been defined above.

The generation of action potentials is described by the following two assumptions:

(A1) All impulses are generated in particular segments which denote the impulse generation area (segment 2 in Fig. 1).

(A2) An impulse is generated when the membrane potential exceeds time-independent firing threshold.

When an action potential is generated, it will normally propagate both along the axon, and backwards towards the dendrites. It is generally assumed that the firing threshold of the membrane in most neurons increases towards the distal parts of the dendrites. If this threshold is sufficiently high, the effect of excitation in nearby segments of the cell is not strong enough to propagate the action potential. Accordingly, areas with sufficiently high thresholds the influence from the firing of the cell is restricted to passive conduction. In the model cell this is described in the following way:

(A3) The cell is divided in active and passive compartments. The active compartment fire automatically at the impulse generating area, while the passive part of the cell is influenced solely through passive conduction.

In a period surrounding the firing, the membrane potential in the active parts of neurons will be determined by the high permeabilities for Na and K. The membrane potential will accordingly be independent of influences from other parts of the cell. The exact form of the time variations in the membrane potential in this period is unknown. Different forms of these time variations were therefore tested. A simple description with no after hyperpolarization phase proved sufficient for most situations.

(A4) After the generation of an impulse in the neuron, the relative depolarisation (V in assumption 1) in the active segments is equal to 0.5 for 1 ms. The membrane potential is afterwards equal to the resting membrane potential for parameter-specified period.

A5 implies that in the period following firing, the membrane potential is kept at sub-threshold level i.e. the cell is refractory. The effect of introducing additional refractoriness absolute or relative by assuming a high threshold in period after firing is very small for moderately long refractory periods. No such period were therefore introduced.

The effect of synaptic activity in chemical synapse is to change the conductance for one or more ion species. In our model this correspond to changes in G_{ij} in assumption A1. More precisely we will assume:

(A6) The change in conductance in one compartment caused by an impulse reaching synapse in that compartment at time t_0 is given by

$$G_{ij}(t) = G_{ijmax} \exp(-\mu(t - t_0)) \quad t > t_0$$

G_{ijmax} and μ are parameters that may vary between synapses. The conductance changes from several impulses are summed linearly.

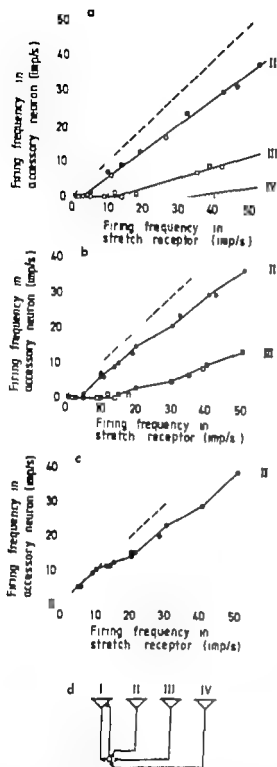


Fig. 2. (a) Firing frequency in an accessory neuron as a function of the stretch receptor input. Open circles, input from one of the two nearest neighbours (II in part d of the figure). Open squares, input from the second nearest neighbour (III in d). The straight lines indicate generalization from this and other experiments. (b) The firing frequency in an accessory neuron as a function of the stretch receptor input. Circles, input from the nearest neighbour. Squares, input from the second nearest neighbour. Open symbols, empirical results. Filled symbols, best fitted model. (c) As Fig. 2b, but from the other side of the same animal. The

The time course of the conductance changes is to some extent arbitrarily chosen. The assumption corresponds to the expected time course if the conductance is assumed to be proportional to the number of activated transmitter receptors. If a certain number of receptors are activated immediately when an impulse reaches the synapse if no receptors are activated later and if the probability of deactivation of an active receptor is constant. Except when μ is small (which corresponds to long duration of the conductance change) small changes in the time course have negligible effects on the behaviour of the model neuron.

Stochastic elements in the transmitter releasing process was introduced in one version of the model by setting $G_{max} = k \cdot N$ where k is a constant and N is a Poisson or binomial random variable representing the number of released synaptic vesicles. Time may be introduced in the release process by describing it as a time dependent Poisson process.

Facilitation was introduced by making G_{max} dependent on the time interval since preceding impulses. An approximation to results obtained by Mallart & Martin (1967) and Magleby (1973) was achieved by making G_{max} proportional to $A_0 + A \cdot \sum_{i=1}^n \Delta t_i$ where A_0 , A and Δt are parameters and Δt is the time interval since preceding impulses.

A computer version of the model was written in the programming language SIMULA 67 and run on the CDC Cyber 74 computer at the University of Oslo. Different experimental situations could then be simulated by activating synapses in a time pattern corresponding to the expected synaptic activity in the experiments (including different levels of uncontrolled spontaneous input).

At first impression of the behaviour of the model neuron may be obtained by looking at Fig. 1. The time course in three compartments of an EPSP caused by an impulse to the 7th compartment (Fig. 1) is shown. Note especially the change in the time course as we move away from the 7th compartment and the effect of a firing including the memory mediated by the passive compartments (compartments 6–10 and 1–16 were passive in this simulation).

RESULTS

Experimental results

It was a typical result that the firing frequency of the stretch receptor had to exceed a threshold before the accessory cell started firing. When the frequency of the input exceeded this threshold the response increased approximately linearly with the input (Fig. 2a). When the stimulus derived from more distant receptors, the threshold was greater and the increase above threshold was smaller. In

simulation results in b and c were obtained with identical parameter values in the model except that the level of spontaneous input was increased from b to c. (d) Schematic drawing of the neuron network.

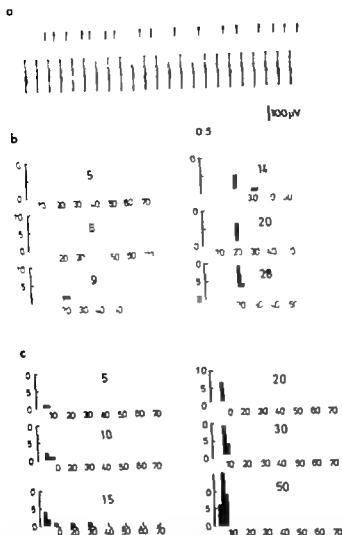


Fig. 3. (a) Reflex activation of an accessory cell. The lower tracing shows spikes in the receptor axon; the upper tracing shows the resulting spikes in the accessory cell. In this experiment the slowly and rapidly adapting receptors were co-activated at constant rate by pulse-fed electromagnetic stretcher. (b) Distributions of latencies in one accessory cell. When III is activated by the stretch receptor in one of its neighbouring segments. Horizontal bar in each of the 6 panels is time in ms. Vertical axis, number of spikes with the corresponding latency. Numbers above each distribution show the mean firing frequency in the stretch receptor which is activating the accessory cell. (c) Distributions of latencies in simulation expts. on the model. Axes as in b. Note that the distributions shown in b include the conduction time in the stretch receptor axon and in the axon of the accessory cell, while in c it does not.

Fig. 4a the results for receptors numbers II and III are from the same animal while the line for receptor IV is based on experience from other experiments. One implication of these observations is especially important. Since a threshold exists, one EPSP generated by the input from a single stretch re-

ceptor cannot alone exceed the firing threshold of the accessory cell. However this threshold was consistently seen in preparations where the accessory cell might fire at each of 2–3 succeeding input impulses at high stimulus intensities (mechanical or electrical) of the stretch receptor. This behaviour is

difficult to explain without assuming that the cell after a firing still contains information about events before the firing. This motivated the introduction of some sort of memory in the model either in the synapses as facilitation or longlasting transmitter effect or in the membrane by assuming that the firing of an action potential only resets the membrane potential to resting levels in part of the neuron (assumption 4 in the model description).

Another characteristic of the signal transmission through the accessory cells is shown in Fig. 3. A firing in the accessory neuron may usually be ascribed to one specific input impulse (Fig. 3a). Fig. 3b shows the results of measurements of the time between action potentials in the receptor axon and in the axon of the accessory cell. If the approximately constant (but unknown) conduction times are subtracted, the measured values represent the time taken from the synapse of the accessory cell receives an impulse until an action potential is generated. These values will be called transmission times. Two observations on transmission times are essential. First, there are marked stochastic variations in the transmission times. Second, the expected transmission time varies as a function of stimulus intensity, with short transmission times connected to high stimulus intensities.

Some sort of systematic variation between different preparations exists. This is illustrated by the difference between the two sides of the animal shown in Fig. 2b and c. The variation is typically restricted to low stimulus intensities. In some preparations the thresholds in the frequency curves are low (or even absent) and the stochastic variation in the transmission time high. With increasing thresholds the variation in transmission time diminishes.

Simulation results

Fig. 2 shows how the model neuron after adjustment of the parameters reproduced the experimental results on the frequency response of the accessory cell. The fit to the data for stimulation of receptors II and III in Fig. 2b were obtained with equal parameters except for G_{max} in A6 which determines the strength of the synaptic input. Fig. 2b compared with 2c also show how the variation between preparations may be explained by differences in the level of activity in neurons other than the stretch receptors. By increasing the number of spontaneous impulses in the model, the response of

the model neuron changes from a pattern close to the experimental results from the left side of the animal (2b) to a pattern close to the results from the right side (2c). Both in accessory cells and the model neuron a high frequency threshold was associated with a low level of random variations in the transmission times, while the stochastic element were marked when the firing threshold was low.

Fig. 3 shows how the transmission time variations were reproduced. Together with Fig. 2 the figure demonstrates that a close agreement between empirical and simulated results may be obtained. However, since the model includes a large number of parameters, good agreement might have been obtained even if the model cell was far from analogous to the accessory cells. A primary examination of whether the close agreement was a multi-parameter artifact was obtained by looking at other aspects of the results from the same experimental situation. The length of the transmission time was for instance in a systematic way dependent on whether or not the preceding impulse from the stretch receptor had fired the accessory cell. The model neuron behaved in the same way and only small additional adjustments of the parameters were necessary to obtain a good agreement (Fig. 4).

Signal transmission mechanisms in the model neuron

The firing pattern of a neuron is determined by the variation in the membrane potential in the impulse generating area. The effect of a certain nerve mechanism is accordingly mediated through its effect on the membrane potential in the impulse generating area. In the following we will therefore first describe characteristics of the synaptic potentials in the impulse generating area, and then look at the mechanisms responsible for these characteristics.

The synaptic potentials had to be of long duration, at least 40–50 ms, from the start until the depolarization was insignificant. Furthermore, the rise-time (the time from the start of the depolarization to the maximal depolarization) had to be long, approximately 10 ms. The amplitude of the potential varied in simulations of different preparations, but for impulses from stretch receptors in the nearest neighbouring segments, the amplitude seemed to be approximately 8–0.9 times the difference between the firing threshold and the resting membrane potential. The rise-time could be deter-

measured relatively accurately while the total duration to a larger degree was dependent on other parameters.

The long duration of the synaptic potentials in the model cell caused by a small resting conductance relative to membrane capacitance (i.e. G_0/C is small in A1). The long rise-time is caused by a considerable electrical distance between the synapse and the impulse generating area (see Fig. 1 and for more thorough discussion of distance effects, Rall 1964).

Memory mediated by depolarisation in the passive compartments gave thresholds combined with a stable linear form of the frequency curve for high stimulus intensities. A relatively large part of the neuron (measured in electric capacitance) had to be passive. The best fit to the experimental data was obtained when approximately half of the compartments were passive.

To understand the variations in transmission times, one has to look at the combined effect of the spontaneous impulse activity and the long duration of the synaptic potentials. The slow EPSP's from the stretch receptors bring the membrane potentials close to the firing threshold for a long period. A extra impulse in this time interval will then fire the cell. In this way we obtain a system where an impulse from a stretch receptor causes a high probability for firing, but where the exact moment for firing is often determined by the additional synaptic input. Accordingly we obtain a randomisation of the firings.

The simulations indicated that the spontaneous impulse activity was fairly high in most experimental situations. Some of the impulses had to generate relatively large synaptic potentials with amplitudes of at least $\frac{1}{2}$ of the firing threshold in the impulse generating area. The time pattern of the spontaneous activity was not important as long as the impulse trains were somewhat irregular and not strongly correlated with the activity in the stretch receptors.

The long rise-time of the synaptic potentials creates an additional stochastic component in the transmission times. If the membrane potential is high before the new impulse from the stretch receptor arrives, only a short time will elapse before the new EPSP has brought the membrane potential above the firing threshold. A low initial membrane potential will cause a correspondingly longer transmission time. If for instance no action poten-

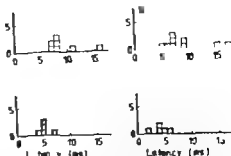


Fig. 4. Distribution of latencies as obtained from an accessory cell (L) and from model simulation (L). Hatched squares. The cell was not fired by the preceding EPSP. Open squares. The cell was fired by the preceding EPSP.

tial is generated after an impulse from a stretch receptor the membrane potential will remain high when the next impulse arrives. Accordingly the transmission time will be short as shown in Fig. 4.

Intracellular recording from axons of abdominal ganglia

We have now shown that the model can reproduce certain aspects of the results in one experimental situation. In order to explore the similarity between the model and the experimental system further we tried to obtain intracellular recordings from the large accessory neurone. Its cell body has been located to the center of the ganglion lying posterior to the one from which the axon emerges (Eckert 1961; Wine & Hagiwara 1977). No neuronal somata impaled in this region showed any synaptic potentials. However processes impaled in the neuropile frequently showed spontaneous excitatory and inhibitory synaptic potentials of several mV amplitudes (see also Calabrese & Kennedy 1974).

The resting membrane potentials in these processes were between 40 and 70 mV but overshooting action potentials were not seen except in a few recordings which appeared to be from axons. Although processes which showed excitatory synaptic potentials correlated with stretch receptor firing were impaled in a large number of preparations spike activity characteristic of the accessory cell was only seen once (Fig. 5). Unfortunately the neurone impaled in this case could not be identified by testing its inhibitory action on the stretch receptor firing (Eckert 1961; Jansen et al. 1970) since conduction in the accessory cell axon blocked accidentally in the second root in this preparation.

Several of the neurons impaled showed parts of

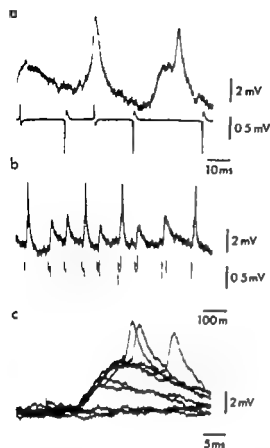


Fig. 5. Intracellular recording from a neuronal process in the neuropile. (a, b) Simultaneous recording of the membrane potential in the neuronal process (upper trace) and the spike activity in the ipsilateral intact second root of the next anterior ganglion (lower trace) during stretch, activation of the slowly adapting receptor (large spikes). The small spikes in the nerve record shows a reflexly activated motoneurone. (The spike of the large accessory cell while initially present, was lost prior to this recording together with the reflex inhibition of the receptor, presumably because of nerve injury. (The site of extracellular recording.)) (c) Electrical stimulation of the second root. The short-latency subthreshold EPSP was produced by the rapidly adapting receptor, and the second EPSP by the slowly adapting one, as confirmed by natural action. Strongly attenuated action potential occurred with variable latency. The resting membrane potential was close to -50 mV. Several sweeps were superimposed at different stimulus intensities.

the behaviour which according to the simulations should characterize the accessory cells, e.g. long-lasting synaptic potentials or passive membrane. In the impalement shown in Fig. 5 the overall behaviour was very close to that predicted for branches of the accessory cell.

— The synaptic potentials had a very long time course which approximated the form in the model cell (compare Fig. 5 with Fig. 1).

— The membrane potential in the recording area was not reset by the action potentials, i.e. this region of the membrane was passive.

— EPSPs with significant amplitudes from spontaneously active neurons were recorded.

Accordingly the intracellular recordings showed that there are ganglion cells innervated by the stretch receptor with the properties predicted in the simulations. The usefulness of the accessory cells for more detailed studies on neuronal mechanisms is, however, seriously restricted by the difficulties of obtaining intracellular recordings.

DISCUSSION

Rall (1964) has shown through theoretical considerations that the form of a synaptic potential may undergo significant changes during the spread through the dendritic area. The present investigation on the accessory cells showed that in this neuron the geometry has marked effects on the transmission properties of the cell. The effects were mediated by at least two mechanisms. First the changes of the form of the synaptic potential during the spread from the synaptic area include a much prolonged rise time. This contributes to the stochastic variation in the times of firing of action potentials. Furthermore, we have shown that the cell may be divided into two parts: in one area the membrane is active, in the other it is passive. As described above, the passive membrane prevents a memory of earlier impulse activity which is essential for the frequency response of the neuron. The passive membrane also seems to have a linearizing effect on summation of several inputs. Accordingly, the function of the accessory cells is a good illustration of how the geometry of a neuron affects its function.

Long-lasting effect of the transmitter would increase the duration of the synaptic potential and might even represent a form of memory after a firing. The simulations could separate the effect of this form of memory from other possible mechanisms, and long-lasting transmitter-effect could be ruled out as the essential memory mechanism in the accessory cells. However, it was not possible to determine whether a long-lasting transmitter-effect was part of the explanation of the long duration of synaptic potentials.

As response to a regularly spaced input from a stretch receptor, the accessory cell will produce an

irregular train of impulses. According to the simulation results most of this irregularity is caused by the combined effect of long-lasting EPSP's from the stretch receptor and spontaneous input from other neurons. If the number of synaptic vesicles released per impulse is small, this might provide an alternative source of stochastic variation. It was however not necessary to add this mechanism to explain the experimental results, and it was not a probable mechanism for explaining the long transmission times found in most preparations at low stimulus intensities.

Small changes in the properties of the accessory cells could reduce its randomising effect, but it then looks as if the cells are constructed for maximizing the randomisation (of course under some constraint). This may seem unfortunate since we normally expect that regularly spaced input would have the highest information carrying capacity. However a closer look at the system reveals that a regularly spaced input from the accessory cells may cause phase-locking in the stretch receptor (Perkel et al. 1964; Læstøl, in prep.). The peculiar effect of this is that for certain frequency shifts in the accessory cell, the high frequency of inhibitory impulses may have a lower inhibitory effect than the low frequency. A way to avoid such phase-locking is randomisation. The randomisation mechanisms in the accessory cells may therefore represent suitable adjustment for avoiding destructive phase-locking.

Facilitation represents an alternative explanation of the memory of earlier synaptic activity after a firing. Since several different mechanisms may produce facilitation, statements about the importance of facilitation in the accessory cells must be made with caution. By introducing facilitation according to the description given by Matsumoto & Martin (1967) and Magleby (1973) in our model frequency thresholds could easily be obtained. However for stimulus intensities above threshold facilitation caused the frequency curve to bend sharply upwards, creating clearly non-linear curve. Even combined with synaptic depression it seems unlikely that facilitation would create stable linear frequency curve like the one observed in our experiments. In contrast, memory caused by depolarisation in the postsynaptic compartments gave thresholds combined with remarkably stable linear form of the frequency curve for higher stimulus intensities. Facilitation was also studied in a set of experiments

where the accessory cells were stimulated alternately by pairs of impulses from one and two stretch receptors. The results supported the conclusion that no significant facilitation was present in these synapses (in contrast to the pronounced facilitation found in many neuromuscular synapses in the crustacea, see i.e. Frank 1973).

The applicability of simulation methods will depend on several factors. Here we shall only discuss the importance of the amplitude and time-course of the synaptic potentials. If we compare the results of our investigation with the results of other similar studies such as the examination of DSCT-neurons in cat by Walløe et al. (1969) some marked differences appear. In order to describe these differences we must first give a brief summary of some results on the summation properties of the accessory cells and the DSCT-neurons. Examination of the summation properties of the accessory cells was conducted by stimulating the cells with sequences of pairs of impulses alternatingly from one or two stretch receptors. In our context the interesting result is that the simulation studies showed that the response of the cell depended significantly on the geometric structure of the neuron, and on the relative position of the synapses. Accordingly the structure is expressed in the cell's response and inference about this structure may be made from the electrophysiological results. On the other hand it is difficult a priori to predict the summation properties of the accessory cells. In some sense the DSCT-neuron represents the converse situation. In DSCT-neurons the synaptic potential is smaller, 3-4 impulses are needed simultaneously to fire the cell. The exact form of the synaptic potentials will then be of less importance, due to the summation of several potentials, and the output signal will contain little information about the geometrical structure of the neuron. In the studies of Walløe et al. the problem was to reveal unknown properties of the input to the neuron. A relatively simple one-compartment model was used with success. Amongst other things it was possible to determine the number of muscle spindle afferents converging on each DSCT neuron and the importance of this number to the transmitting properties of the cell. In general, a neuron with large synaptic potentials such as the accessory cells or for example cells in mammalian sympathetic ganglia, will represent a problem in situations where description of summation properties is needed but an advantage if the

purpose is to examine other inherent mechanisms of the neuron

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COMMUNICATIONS

Influence of the Na⁺-K-activated ATPase on resistance vessel activity in normotensive rats compared with a model of primary and secondary hypertension

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To investigate the role of Na⁺-K-activated ATPase in the regulation of resistance vessel activity, paired perfused hindquarters of Wistar-Kyoto (WKR) and spontaneously hypertensive (SHR) and two kidney-one clip renal hypertensive rat (RHR) Ouabain was utilized to inhibit the sodium pump in vascular smooth muscle.

Ouabain up to a perfused concentration of 10^{-4} M did not alone noticeably enhance vascular tone in the present situation. Relatively low constant flow Bouve vasocostrictor of fentanyl (NA) and isoprenaline (IP) and barium chloride (Ba^{++}) were only tentatively enhanced by ouabain, whereas maximal gonit effect had already been achieved. ED₅₀ values before and after ouabain addition were computed from the log dose-response curves of various agonists, and the shift caused by ouabain is referred to as ouabain sensitivity index (OAI).

OAI ED₅₀ of gonit/ED₅₀ of gonit ouabain. There was no significant OAI difference between WKR and RHR. In SHR, however, OAI was significantly greater than in normotensive rats, indicating an increased sensitivity of the ouabain-sensitive sodium pump in smooth

muscle of the SHR. In these vessels, the increase in RHR was less than in paired difference. OAI of Ba^{++} did not differ significantly between RHR and normotensive controls.

Based on these findings, the following conclusion may be reached:

1. The responsiveness of resistance vessel constriction to a considerable degree is influenced by the activity level of the Na⁺-K-ATPase.
2. In SHR resistance vessels, the sodium pump activity may be increased, perhaps as a compensatory reaction to elevated passive sodium permeability. The OAI alterations in RHR represent secondary hypertension, whereas the agonist used and may reflect a smooth muscle relaxation difference from that in SHR primary hypertension.
3. The exaggeration of an increased sodium pump activity in SHR vascular smooth muscle is in keeping with observation by their investigators on erythrocytes from SHR and sensitive hypertensive patients indicating an increased passive sodium permeability that is partly offset by accentuated pump activity.

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Vibrations cause electro-mechanical uncoupling in the spontaneously active longitudinal muscle of the rat portal vein

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In previous studies from this laboratory vibrations have been found to reduce active force development in isolated vascular smooth muscle. There is indirect evidence that this inhibition is caused by a direct action on the contractile process (Ljung & Sivertsson 1975, 1980).

In order to elucidate the effects of vibrations on the electrical membrane discharge and on the contractile force of the spontaneously active longitudinal smooth muscle of the isolated rat portal vein was studied. The vein was mounted vertically in an organ bath filled with modified Krebs solution between a submerged isometric force transducer and a vertical rod extending from a servo-controlled vibrator. The electrical activity was recorded extracellularly. The rectified signal was integrated over 1 min periods and related to the mean isometric active force developed over the same time period. Sinusoidal vibrations (40 Hz, 2.5-30 % tissue length peak to peak) applied in the longitudinal direction of the smooth muscle caused prompt and reversible reduction of the active force. In contrast, neither the pattern of the phasic contractions nor the membrane discharge were altered. The amplitude of the active force decreased with vibration amplitude being 50 % of control at a vibration amplitude of 12 ± 4 %

(mean \pm SD, n = 8).

Oscillations with characteristics corresponding to those of the vibrations applied in the present experiments may prevail in peripheral blood vessels in various occupational situations as well as under pathophysiological and possibly physiological circumstances.

It is concluded that vibrations of vascular smooth muscle cause dissociation of electrical membrane discharge and mechanical force development. This finding of electrical-mechanical uncoupling provides further support to the previously forwarded hypothesis that vibrations cause inhibition of smooth muscle contraction by a direct action on the contractile mechanism. It is likely that the induced length changes lead to increased rate of detachment of the actin-myosin cross-bridges.

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influence of initial microvascular resistance on the magnitude of a superimposed myogenic microvascular constrictor response; importance of physical factors

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It has been suggested by Myers and Honig (1968) that the magnitude of response of the resistance vessel to a given vasomotor stimulus is increased with increasing level of vascular tone prior to the stimulus. This is in agreement with previous study from our laboratory (Grende 1979) demonstrating that the magnitude of dynamic myogenic constrictor response to standardized transmural pressure stimulus was significantly and gradually increased with increasing level of microvascular tone.

The present study attempts to estimate the importance of possible physical factors both to reinforce the phenomenon by making corresponding analysis using only previously published mathematical model for the microvascular bed. The model was based on force-equilibrium, the vessel wall included passive forces related to vascular transmural pressure, and its activity and active myogenic forces assumed related to wall tension and its rate of change (for details of the model see Borgstrom & Grende 1979). This analysis demonstrates great resemblance between the dynamic microvascular resistance responses obtained with the model and corresponding curves observed in vivo and indicates the reinforcing phenomenon to be mainly ascribed to an increase in gain implicit in the inverse fourth power relationship between microvascular resistance and internal vessel radius according to Poiseuille's law though counteracted by effects of Laplace law and to some extent by concomitant effects of shift along the smooth muscle length-tension curve.

It can be concluded that physical factors significantly must add to the complexity in overall vascular control and that the radius factor in Poiseuille's law per se inevitably leads to vast deviation from linearity of circulatory resistance regulation. The phenomenon could also bear some relationship with the increased reactivity in hypertensive compared to normotensive rats which according to the Folkow hypothesis mainly can be explained by structural increase of wall lumen ratio (see Folkow et al 1970).

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C4

Plasma colloid osmotic pressure in venous blood from the foot of man in the sitting position

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Flow to of edema formation in the legs from standing or sitting quietly has attracted the interest of physiologists for many years. Increased interstitial hydrostatic pressure and increased area available for filtration are among the explanations of edema. Hollander & Oberg (1964) deduced from observations of the filtration coefficient that the filtration area was greatly reduced due to closure of precapillary sphincters following changes from the supine to the erect position. The capillaries would however be expected to remain open when the venous pressure is high even if precapillary sphincters are closed. Our data from humans vital still support the venous plasma osmotic pressure as the key-down position. We wished to follow up and expand this pioneer study. A low flow through the vascular bed could cause the elimination of lymphatic fluid and increase in plasma colloid osmotic pressure and even to filtration because the increased colloid osmotic pressure could partly counter-balance the increased capillary pressure. An increased plasma colloid osmotic pressure would cause an error in the calculation of the filtration coefficient because the coefficient is usually calculated with the net filtration pressure

increases in proportion to the venous pressure in the seated relaxed human comfortable environmental temperature with the foot being about 40 cm below heart level we found the colloid osmotic pressure in plasma from blood sampled from veins on the dorsum of the foot to be about 5 mmHg higher than in plasma from the arm. This measurement probably grossly underestimated the increase in capillary colloid osmotic pressure due to admittance of blood flowing through arterio-venous anastomoses. We repeated the experiment with the subject in cold environment and now the measured colloid osmotic pressure was about 15 mmHg higher in blood from the foot than from the arm. We do not know however to what extent admittance of blood flowing through arterio-venous anastomoses still took place. We conclude the rise in capillary colloid osmotic pressure may significantly contribute to prevent edema in the legs of the erect immobile human.

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Influence of the Na-K-ATPase on resistance vessel reactivity in normotensive rats compared with a model of primary and secondary hypertension.

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To investigate the role of Na-K-activated ATP-ase in the regulation of resistance vessel reactivity, we perfused hindquarters technique was used in Wistar-Kyoto (WKR) and spontaneously hypertensive (SHR) and two kidney-one clip renal hypertensive rat (RHR). Ouabain was utilized to inhibit the sodium pump in vascular smooth muscle.

Ouabain up to perfusate concentration of 10^{-4} M did not noticeably enhance vascular resistance in the present situation. A low constant flow however was obtained at a flow rate of renal plasma flow (RPF) and basal flow (BF) were consistently enhanced when ouabain was added except when maximal flow rate had already been reached. ED₅₀ values before and after ouabain addition were computed from the log dose-response curves. Vascular resistance and the flow rate by ouabain were related to ouabain activity index (OAI) computed as follows:

OAI = $\frac{ED_{50} \text{ of control}}{ED_{50} \text{ of ouabain}}$
The value of OAI did not differ significantly between WKR and SHR. However, OAI was significantly greater than in normotensive rats indicating an increased sensitivity of the ouabain sensitive sodium pump in smooth

muscle of the SHR resistance vessels. The situation in RHR was at least in part different since OAI for RHR did not differ significantly between RHR and normotensive control.

Based on these findings the following conclusion may be reached:

1. The response of resistance vessels to ouabain is a considerable degree influenced by the activity level of the Na-K-activated ATP-ase.
2. In SHR resistance vessels the sodium pump activity seems to be increased perhaps as compensation for an increased passive sodium permeability. The OAI situation in RHR representing secondary hypertension was not clearly different from that in SHR primary hypertension.
3. The suggestion of an increased sodium pump activity in SHR vascular smooth muscle is in keeping with observations by other investigators on cytochrome c from SHR and sensitive hypertension patients indicating an increased passive sodium permeability that is partly offset by compensated pump activity.

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Vibrations cause electro-mechanical uncoupling in the spontaneously active longitudinal muscle of the rat portal vein

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In previous studies from this laboratory vibrations have been found to reduce active force development in isolated vascular smooth muscle. There is indirect evidence that this inhibition is caused by a direct action on the contractile process (Ljung & Sivertsson 1975, 1980).

In order to elucidate the effects of vibrations on the electrical membrane discharge and on the contractile force, the spontaneously active longitudinal smooth muscle of the isolated rat portal vein was studied. The vein was mounted vertically in an organ bath filled with modified Krebs solution between a submerged isometric force transducer and a vertical rod extending from a servo-controlled vibrator. The electrical activity was recorded extracellularly. The rectified signal was integrated over 1 min periods and related to the mean isometric active force developed over the same time period. Sinusoidal vibrations (40 Hz, 2.5-30% tissue length peak to peak) applied in the longitudinal direction of the smooth muscle caused prompt and reversible reduction of the active force. In contrast, neither the pattern of the phasic contractions nor the membrane discharge were altered. The amplitude of the active force decreased with vibration amplitude, being 50% of control at a vibration amplitude of $12 \pm 4\%$

(mean \pm SD, n = 8).

Oscillations with characteristics corresponding to those of the vibrations applied in the present experiments may prevail in peripheral blood vessels in various occupational situations as well as under pathophysiological and possibly physiological circumstances.

It is concluded that vibrations of vascular smooth muscle cause dissociation of electrical membrane discharge and mechanical force development. This finding of electrical-mechanical uncoupling provides further support to the previously forwarded hypothesis that vibrations cause inhibition of smooth muscle contraction by a direct action on the contractile mechanism. It is likely that the induced length changes lead to increased rate of detachment of the actin-myosin cross bridges.

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The effect of Ca^{2+} on the ionic current in the presynaptic nerve membrane

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The properties of ionophores have mainly been studied on artificial membranes (Haydon 1970). Very little is known of their effects on natural excitable membranes. Our aim with the present study was to study the effects on such natural membranes, namely the membrane at the node of Ranvier.

We studied the effects on the ionic currents in fibers from the clawed toad *Xenopus laevis* by potential clamp method (Dodge & Frankenhaeuser 1958). A number of ionophores were tested but only the crown ether dicyclohexyl 18 crown 6 was found to change permeability parameters.

The most striking effect (in the concentration range 1-100 μM) was an activation of the potassium current associated with positive potential steps. The potassium current decreased exponentially with time constant of about 10 ms concentration of 10 μM .

The effect disappeared gradually after exposure to the holding potential. The time to reach 50% recovery of polarization to 70 mV was about 100 ms at concentration of 10 μM . The recovery time did not depend markedly on the holding potential.

The effect depended clearly on external K^+ concentration. In solutions with high $[\text{K}^+]_o$ the inactivation was pronounced than in solutions with low $[\text{K}^+]_o$. The decay of the outward potassium current was proportionally equally large as the decrease of the inward potassium current associated with polarization after positive potential steps. Thus steady-state was observed.

Some of the findings may be of relevance for the knowledge on the potassium transport system in the nerve membrane. It is often assumed that the system consists of long pores with gating mechanisms at the axoplasmic end (Armstrong 1975). The main conclusion from the present study is that our results do not support such simple model of the potassium system.

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C8

Facilitation of breathing by low threshold lung mechanoreceptors

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Voluntary mediated excitatory effect on phrenic nerve activity elicited by lung deflations and deflations have been repeatedly described. In addition to the facilitatory effect of large increases in flow (1), it has been recently suggested that volume dependent excitatory reflexes also exist (2, 3). Experiments were performed in 11 awake anesthetized cats (pentobarbitone PB) to establish and further characterize the effect of varying volume feedback on inspiratory activity in the phrenic (P) and external intercostal (EI) nerves. P and EI tidal volumes (V) flow (V) tracheal pressure (TP) and P_aCO_2 were simultaneously recorded. Integrated P and EI were compared with and without afferent feedback from lung mechanoreceptors in spontaneously breathing animals (tracheal occlusion) and with varying feedback in paralyzed cats ventilated with servo-pneumatically generated either by phrenic activity or different gain or by phrenic triggered ramp in volume of varying slope and amplitude. The computerized analysis of the data was compared in the range of 5 preceding breaths in lightly anesthetized cats, after varying delay from the onset of inspiration volume change to the excitatory effect on both P and EI. The change in P and EI from control was linearly

related to change in TP and V but not to \dot{V} . This inspiratory facilitation could be evoked by small volume increments at unchanged values of V. The effect occurred continuously in every breath. Small additional doses of PB (4-6 mg/kg) abolished the excitatory effects without impairing the vagal inhibitory effects on inspiratory duration (T_i) so the inspiration facilitating effect of tidal inflation. Although vagotomy eliminated the volume facilitatory effect, excitatory effect could be produced in EI by electrical vagal stimulation at inspiratory threshold strengths equal to produce effects on T_i . Produce excitatory effects on P large stimulus strength was required. P and EI equally responded quantitatively differently to the same volume stimulus in that any given volume change produced larger linear change in EI than in P. After vagotomy EI was then considerably reduced whereas P was little affected.

These results suggest that volume related information from low threshold lung mechanoreceptors can exert an excitatory effect on inspiratory motor neuron output. However, this facilitating effect probably does not involve the R_p neuron in its central pathway as does the inhibitory effect.

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We have recently developed a tiny metabolism chamber including treadmill for the study of exercising rats and shown that before graded exercise test it is necessary to introduce 20 min pre-test period during which unspecific stress responses disappear (Sonnen & Galbo 1980).

The aim of this study was to evaluate the effect of running speed on blood lactate (L) and glucose (G) concentrations on pH, oxygen (P_{O_2}) and carbon dioxide (P_{CO_2}) tensions in arterial blood and on oxygen uptake ($\dot{V}O_2$), heart rate (HR), respiratory exchange ratio (R) and rectal temperature (T_{re}) and to evaluate post-exercise changes in these parameters.

7 rats had a catheter implanted in the left common carotid artery the tip of the catheter being freely floating in aorta. 11 days later they ran at an inclination of 0% and running speed of 10 m min⁻¹ for 30 min. Thereafter the speed was stepwise increased with 5 m min⁻¹ every 10 min. Blood was withdrawn in the 30th, 40th, 20 min after exercise and in the 3rd min after exercise.

L, G, $\dot{V}O_2$, HR, R and T_{re} increased gradually ($P < 0.05$) from 1.12 ± 0.21 mmol l⁻¹ (mean \pm SE) 6.06 ± 0.13 mmol l⁻¹, 4.1 ± 0.2 ml O₂ (STPD) (min 100 g)⁻¹, 4.56 ± 24 beats min⁻¹, 0.76 ± 0.03 and 37.6 ± 0.4 °C respectively. Running speed of 10 m min⁻¹ to 2.62 ± 0.89

7.51 ± 0.71 , 5.7 ± 0.2 (about 45% of $\dot{V}O_{2max}$ for rats) 520 ± 13 , 0.83 ± 0.01 and 38.3 ± 0.2 respectively. Speed of 20 m min⁻¹ the highest speed all rats were able to cope with whereas P_{ACO_2} decreased from 39 ± 2 mmHg to 31 ± 1 . In the 3rd min after exercise $\dot{V}O_2$, HR, $\dot{V}O_2/HR$ and T_{re} had decreased, gas ($P < 0.05$) compared to the last values obtained during work. R, L and P_{ACO_2} had not changed and G had increased further to 8.35 ± 0.69 ($P < 0.02$). P_{O_2} , pH and hematocrit remained unchanged: 110 ± 3 mmHg, 7.42 ± 0.01 and $38 \pm 1\%$ respectively throughout the experiment.

The marked increase in glucose and the small increase in lactate concentration are compatible with the fact that rats have relatively larger liver glycogen stores and smaller muscle glycogen stores than man. The increase in blood glucose concentration during the experiment indicates that in rats hepatic glucose production is not always totally adjusted to peripheral glucose utilization on some time to moment basis. The decrease in $\dot{V}O_2/HR$ after exercise indicates a decreased stroke volume and/or a decreased arterio-venous oxygen difference soon after work.

The rapid post-exercise changes bring into discredit many previous studies of exercising rats using post-exercise sampling.

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pO_2 and pCO_2 in human muscle during exhaustive static contraction

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The probe described by Lundgaard et al. (1980) for measurements of gas tensions in blood was used. The probe consists of a 1 mm stainless tube fitted with porous tip of tapered bronze covered with 20 micron polyethylene membrane. The probe was introduced in a vastus lateralis in 4 subjects through small incision in skin and epidermis. The steel tube was connected to the inlet of a 4-channel gas spectrometer (Micromass). As the pressure in the spectrometer is 10^{-3} Torr, gas will diffuse from the surrounding muscle tissue through the polyethylene membrane. The tensions of Ar, O₂, CO₂ and N₂ were recorded on a channel strip chart recorder. The probe was calibrated in vitro at 37°C in isotonic saline equilibrated with atmospheric air and known CO₂ tension during stirring. The low solubility for O₂ in tissue (0.021 ml/ml/Atm) creates an increased diffusion resistance which attenuates the pO_2 signal from the muscle. However, in vitro experiments showed that the Ar and the O₂ signal were attenuated to the same degree, hence it was possible to calculate the tissue pO_2 because pAr in tissue and air are identical.

Resting pO_2 was 57.8 ± 6 and pCO_2 39.9 ± 7 Torr (mmHg). At exhaustion during static exercise

100%, 50% and 25% MVC pO_2 was 20.4 ± 1.3 , 4.5 and 14.3 ± 0.7 Torr while pCO_2 was 50.3 ± 5.7 , 42.5 and 51.7 Torr. Recovery started within few seconds after exercise for pO_2 while pCO_2 continued to increase for another 50-60 seconds up to 70-90 Torr. Resting value was obtained after 10 min. The resting oxygen consumption rate was calculated to 1.8 ml/kg/min (STPD) from the decrease in pO_2 during resting occlusion. In this situation pO_2 decreased to zero or close to zero within 200 seconds if then static contraction was performed no further change was seen in pO_2 while pCO_2 increased to level of 80-100 Torr. This increase in pCO_2 when no oxygen is consumed is an indication of pH change, pH tending to 7.15 thus liberating CO₂ which is buffered in the tissue.

The *in vivo* technique here presented is promising for the further study of metabolic events in the muscle. It gives reliable results within the range expected for pO_2 and pCO_2 but an alternative method for the study of any of the gas tensions.

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The effect of crown ether on the ionic currents in the myelinated nerve membrane

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The properties of ionophores have mainly been studied on artificial membranes (Haydon 1970). Very little is known of their effects on natural cell membranes. Our aim with the present study was to analyze the effects on such natural membranes, namely the membrane at the node of Ranvier.

We studied the effects on the leak current in fibres from the clawed toad *Xenopus laevis* by potential clamp method (Dodge & Frankenhaeuser 1958). A number of ionophores were tested but only the crown ether dicyclohexyl 18-crown-6 was found to change permeability parameters.

The most striking effect (in the concentration range 1-100 μM) was an inactivation of the potassium current associated with positive potential steps. The potassium current decreased especially with time constant of about 10 ms at a concentration of 10 μM .

The effect disappeared gradually after exposure to the holding potential. The time to reach 50% recovery of polarisation at 70 mV was about 100 ms at a concentration of 10 μM . The recovery time did not depend markedly on the holding potential.

The effect depended clearly on internal $[K^+]$. In solutions with high $[K^+]$ the inactivation was pronounced than in solutions with low $[K^+]$. The decrease of the outward potassium current was proportionally equally large as the decrease of the inward potassium current associated with depolarisation after positive potential steps. Thus rectification was observed.

Some of these findings may be explained if the knowledge on the potassium transport system in the nerve membrane is taken into account that the system consists of a leak pore with a gating mechanism at the extracellular end (Armstrong 1975). The main conclusion from the present study is that our results do not support such a model of the potassium system.

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C8

Facilitation of inspiration by low frequency breathing

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Typically mediated excitatory effects on phrenic nerve activity elicited by rapid inflation and deflation have been repeatedly described. In addition to the facilitatory effect of large inflation as in flow (1), it has been widely suggested that volume dependent excitatory effects also exist (2, 3). Experiments were performed in 11 anaesthetized cats (pentobarbitone PB) to establish the characteristics of the effect of varying volume feedback on inspiratory activity in the phrenic (P) and external intercostal (EI) nerve. P and EI tidal volume (V) flow (V̇) tracheal pressure (TP) and $P_a\text{CO}_2$ were simultaneously recorded. In 8 of 11 cats P and EI were compared with and without afferent feedback from lung movements in spontaneously breathing animals (tracheal occlusion) and with varying feedback in paralyzed cat ventilated with servo-spirator governed either by phrenic activity at different gains or by phrenic triggered ramp in relation of varying slopes and amplitudes. The computer averaging of 5 test breaths was compared to the average of 5 preceding breaths. In lightly anaesthetized cats, after varying delay from the onset of inspiration, volume change elicited an excitatory effect on both P and EI. The change in P/EI from control was linearly

related to change in TP and V but not to V̇. This inspiratory facilitation could be evoked by small volume increments at unchanged values of V̇. The effect occurred continuously in every breath. Small additional dose of PB (4-6 mg/kg) abolished these excitatory effects without impairing the vagal inhibitory effects on inspiratory duration (T_I) nor the inspiration facilitating effect of rapid inflation. Although vagotomy limited the volume facilitatory effect excitatory effects could be produced in EI by electrical vagal stimulation at smaller threshold strengths equal to threshold effects on T_I. T_I produced excitatory effects on P larger stimulus strengths were required. P and EI usually responded quantitatively differently to the same volume stimuli in that any given volume change produced larger latency change in EI than in P. After vagotomy EI was often considerably affected whereas P was latently less affected.

These results suggest that volume related information from low threshold lung mechanoreceptors can exert an excitatory effect on inspiratory motor neuron output. However, this facilitating effect probably does not involve the R₁ neurons in its central pathway due to the inhibitory flex.

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Afferent A and C fibre interaction in the baroreflex

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Input from arterial baroreceptors are transmitted to the brain via myelinated (A) and non myelinated (C) fibres. The A and C fibres differ in receptor thresholds and discharge frequencies but reflex responses to activity in the fibres are roughly the same: bradycardia and a fall in arterial pressure. The aim of the present study was to examine whether afferent signals in A and C fibres show interaction in their reflex effects or whether they are subjected to simple addition.

The rabbits were anaesthetized with chloralose and urethane. With other baroreceptor nerves intact the central end of the cut left aortic nerve was stimulated with two sets of electrode: one of excitation of A fibres (pulses of 60-100 Hz, 0.2 ms and 5 V) and one for activation of C (and A) fibres (2-4 Hz, 1 ms, 6 V). The pulse rates in C stimulations were too low to evoke reflex effect via A fibres. Several stimulation series consisting of separate A and C fibre stimulations and a combined stimulation were run in each animal. Pulse frequencies were varied from one series to another in order to obtain a range of low to medium reflex responses.

The hypotensive response to combined stimulations of A and C fibres (A+C) was found to exceed the sum of responses to separate A and C stimulations in 21 of 22 series. For whole-nerve activity in the left renal nerve a greater than additive effect was observed in 16 of 24 series (P = 0.08) while in six of the series the responses were equal. Median values of the ratio $AC/(A+C)$ were 1.28 and 1.11 respectively. Similarly C stimulations which in comparison to A stimulations affected sympathetic activity to the kidney relatively more than blood pressure in 13 of 16 series (P = 0.01) produced a greater sympathetic inhibition when added to a background of A fibre activity than when alone. Changes in heart rate were minimal.

Others have found a positive interaction between reflex responses to stimulation of all fibres in ipsilateral aortic and carotid sinus nerve (Kendrick, Matson & Malley 1979). The present results show that a synergistic interaction may exist also between reflex responses to activity in different fibre populations within one baroreceptor nerve.

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C 10

INFANT LOCOMOTION an inherited motor program for locomotion?

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Throughout the animal kingdom it has been shown that locomotion is generated by central motor programs genetically encoded for each species (eg Wilson 1961; Grillner & Zangger 1979). An hierarchical organization has been revealed in mammals as a significant part of the central network is located in the spinal cord (Brown 1911; Grillner & Zangger 1979) and as the spinal networks together with reflex interaction can produce a locomotor pattern that even in many details resembles that of intact animals (Forssberg et al 1980).

There is today no possibility in a direct way to explore how the neural control of locomotion is organized in humans but the aim of the present investigation is to study the development of human locomotion to see if it is in accordance with similar central and hierarchical organization. The human starts to walk without external support about one year after birth but already at birth locomotor-like activity can be induced if the infant is held erect with the feet on a flat surface (Andre-Thoms & Autsgarden 1966). This innate locomotion has been analyzed in detail in 4 infants and by recordings of movement, muscular activity and force reactions. The locomotion is irregular and compared to adult locomotion there are significant differences such as a pronounced hip flexion, a variable digit 1 or plantigrade foot contact, a premature activation of the calf muscles (before

foot contact) and a high degree of cocontraction between antagonistic muscles. When the children learn to control their locomotion (ie walk independently) the pattern successively develops to be more like the adult locomotion but still many of the variations from the infant locomotion be seen (see also Statham & Murray 1971).

Of special interest is that certain of the differences are similar to those seen when the adult bipedal gait is compared to the quadrupedal one, in which only the toes contact the ground (digitigrade). It is therefore suggested that humans are born with an innate network for locomotion that is programmed for a quadrupedal gait. The ontogenetic development should then reflect the phylogenetic evolution to a bipedal gait due to modifications of the central motor program.

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fractional and morphological evidence that small resistance vessels in human omentum have little adrenergic innervation

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The quantity of adrenergic innervation of arteries varies between different regional circuits as well as between consecutive vascular segments (Folkow & Hell 1977). We have investigated the amount of adrenergic innervation of small human arteries (internal diameter $1.0 \pm 0.200 \mu\text{m}$) from the omentum as it has been shown earlier (Stjärne & Brundin 1975) that larger arteries in this circuit (internal diameter $\approx 1.1 \text{ mm}$) have adrenergic innervation.

Segments of 19 human omental arteries were mounted on a myograph which permits precise measurements of wall tension while the internal circumference can be controlled (Mulvany & Halpern 1978). After mounting the media thickness was measured by light microscopy ($8.60 \pm 0.50 \mu\text{m}$). The wall tension response to K-depolarisation ΔT_w was measured ($2.02 \pm 0.15 \text{ mN/mm}$) and the magnitude of this response was not affected by phentolamine ($1 \mu\text{M}$). Cumulative noradrenaline (NA)-dose-response curves showed NA-ED of $0.415 \mu\text{M}$ (NA- $\text{pD} = 6.39 \pm 0.30$) and a maximum response of $4.4 \mu\text{M}$ NA of $1.60 \pm 0.35 \text{ mN/mm}$. This maximum response corresponds to an active media stress of $196 \pm 25 \text{ mN/mm}^2$ which is an indicator of the viability of the preparation. The NA-response was totally inhibited by phentolamine. Addition of $1 \mu\text{M}$ cocaine (inhibiting the active pump) to any adrenergic nerve-terminal had no significant influence on the maximum response nor did it produce

any significant shift of NA-ED (ED₅₀ ratio = 1.16, 0.31). Similar experiments using two large omental arteries (i.d. $518 \mu\text{m}$ and $752 \mu\text{m}$) showed a greater wall tension response ($\Delta T_w = 95 \text{ mN/mm}$ and 10.44 mN/mm) and a shift of the NA-ED to smaller values with cocaine (ED₅₀ ratio = 2.0 and 1.77). On completion of the mechanical experiments some of the vessels were prepared for fluorescence microscopy. This showed that the $200 \mu\text{m}$ vessels had very little or no innervation whereas the two "large" vessels were richly innervated. As further control mesenteric arteries (i.d. $150\text{--}200 \mu\text{m}$) from Wistar-Kyoto rats were investigated. These vessels showed very rich innervation with fluorescence microscopy as well as a cocaine-induced left shift of the NA-ED (ED₅₀ ratio = 3) and considerable reduction (about 50%) of the K-response with phentolamine.

The lack of a left shift of NA-ED with cocaine indicates a very low active pump activity in the vessel wall. The lack of effect of phentolamine on the K response indicates very little or no norepinephrine release of NA during K-depolarisation. These findings together with the scanty fluorescence of the $200 \mu\text{m}$ omental vessels give functional as well as morphological basis to conclude that these vessels have very little adrenergic innervation. It is therefore unlikely that these vessels play a significant role in the sympathetic control of blood pressure.

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Adrenergic and/or interaction with the constant response in the vessel of the skeletal muscle during hemorrhage

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Such effect has been devoted to the problem of regulation during hemorrhage of peripheral vascular resistance. Hemodynamically important vascular areas such as skin and skeletal muscle. The present, however, has most studies been focused on the vasoconstrictor mechanisms brought into action in response to bleeding. Little attention being paid to the possibility that autospinal dilatation in turn with the vasoconstrictor influence may be of significance in determining the net resistance effect. The present study describes quite pronounced β -adrenergic dilatator influence on skin and skeletal muscle during reflex sympathetic-adrenal activation evoked by graded hemorrhage. The dilatator effect was revealed in terms of much greater increase of vascular resistance in the skin bed with blocked β -adrenoceptors (propranolol) than in the vascular bed in which the adrenoceptors were left intact. In both tissues the β -adrenergic dilatator influence was graded in relation to the level of hypotension and to severe bleeding (hemorrhage) hypotension of 50 mm Hg vascular resistance was usually about twice as high after than before β -blockade. In some preparations

the β -adrenergic effect was fact much more pronounced. Thus, it is well established so that blood flow almost ceased after administration of propranolol.

The β -adrenergic dilatator mechanism may serve to improve nutritional blood flow by counteracting the α -adrenergic constrictor response. Further study contributes to determine the distribution of cardiac output during reflex sympathetic-adrenal activation.

HYDRAULIC CONDUCTIVITY OF THE PERITUBULAR CAPILLARY MEMBRANE IN THE RAT KIDNEY N LAUSON

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The hydraulic conductivity of the peritubular capillary membrane was determined from 1. The net glomerular fluid absorption and 2. The net driving force for the hydrostatic and colloid osmotic pressure in the interstitium and peritubular capillary blood as investigated with micropuncture technique and computerized model.

During control antidiuretic conditions the net driving force was estimated to 13.4 mm Hg and the hydraulic conductivity to 0.04 ml/(min mm Hg) per 100 g. At during 4% and 10% saline expansion the net driving force decreased to 12.5 mm Hg and 6.4 mm Hg respectively whereas the conductivity increased to 0.63 and 0.14 ml/(min mm Hg) per 100 g rat. The reduction in net driving force was thus at least partly compensated by an increased hydraulic conductivity in the glomeruli. The net driving force for filtration increased from 14.2 mm Hg during control conditions to 21.2 mm Hg and 25.3 mm Hg during 4% and 10% saline expansion whereas the corresponding hydraulic conductivity decreased from 0.13 l/(min mm Hg) per 100 g to 0.03 and 0.00 ml/(min mm Hg) per 100 g during the two expansions.

These changes in the hydraulic conductivity will cause retardation of the exchange fluid during expansion but then also prolongation of the time for the filtration process.

open to this is in favour of the renal adaptation of toxic substances and metabolic end-products. From the mass transport of albumin and insulin the effective pore radius was calculated to range between 50-70 Å. Using the Poiseuille equation the permeability term $A/4l$ the total pore area over the pore length could be evaluated. The results show that the total pore area is very large at about 1% of the total capillary area. During 4% and 10% saline expansion the total pore area will increase to 1.8% and 3.2% for the two expansions respectively. It is supposed that the increased total pore area during expansion is caused by the stretching of the vessel wall which is true due to an increase in the osmotic pressure difference from 6 mm Hg during control conditions to 12.8 mm Hg during 10% saline expansion.

C 14

Effect of sodium/angiotensin infusion on fluid balance in the rat
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Angiotensin II (A II) infused into the dorsal aorta of the rat (10 µg/kg/h) has been found to interact with the renin-angiotensin system (RAS) in the brain. This interaction is of a bidirectional nature (as reported in a preliminary report and in a paper by Andén et al., 1977). A study of the antidiuretic and diuretic effects of the interaction has also been demonstrated with a bidirectional relationship in the amounts of A II (Lek et al., 1977). However, the effect of the bidirectional relationship on the interaction between CSF Na and A II has not been investigated.

To obtain a direct quantitative measure of the effect of Na/A II interaction on the RAS (0.02 ml/min) of a) 25 mM NaCl (N 10) b) A II (0.3 µg/kg/min) in isotonic NaCl (N 10) and c) A II (dose as b) in 0.25 M NaCl (N 9) were made into the total cerebroventricular fluid of two hydrated rats. During the preinfusion control period a glomeral osmotic pressure (AVP) was not detectable in the interstitium (AVP) was not detectable in the interstitium with the method employed.

The mean (±SEM) amounts of AVP released by radioimmunoassay in the urine in response to the various infusions were 1.5 (a), 2.8 ± 0.7 (b), 3.6 ± 1.3 (c) and 13.3 ± 2.2 (g). The AVP response to the simultaneous infusion of

hypotonic NaCl and A II was approximately two times as large as the sum of the responses to the separate infusions of hypertonic NaCl and A II. The relationship between Na/A II potention and diuresis (the neurophysiological parameters) is likely to be involved in the prehydrated animals whereas the infusions of A II of 0.25 M NaCl did not have any diuretic effect.

The results provide support for the idea (cf Andén et al., 1978) that the bidirectional relationship between angiotensin II and AVP is not mediated by a long-term action of just the cerebrovascular response.

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developmental patterns of PKC ϵ and 5-HT metabolism is isolated perfused fetal and neonatal rabbit lung

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Pulmonary circulation is known to metabolize selectively vasoactive hormones (Bakke & Vane 1974). Very little is known about the development of these processes in fetal and neonatal pulmonary circulation. The aim of the present study was to investigate the development of PKC ϵ and 5-HT metabolism in fetal and neonatal rabbit lung.

Lung perfusions were made at 28th, 30th and 33rd days of fetal life and on the first, second and seventh day after birth. Fetal rabbits were obtained by caesarean section during spinal anaesthesia and the airways were allowed to breathe spontaneously in prepping lung perfusions. Perforated umbilical cannulae was used. After thoracotomy the main pulmonary artery was ligated and the pulmonary artery was inserted into the pulmonary artery through the right ventricle. The pulmonary vascular bed was washed with 1 ml of saline, and the isolated lung block was connected to perfusion apparatus. Perfusion was made with Krebs-Henseleit (pH 7.4, 37°C) containing 1 g/l glucose at flow of 8 ml/min and perfusion pressure was maintained within physiological limits. Amount of injected PKC ϵ (200-500 ng) was made by measuring with hamster stomach strip and 5-HT (0.4 μ M) by column chromatography.

as described on liver (Bakke, Martiala & Iivonen & Kotila 1979).

The survival of PKC ϵ presented decrease from 11% at 28th day of gestation to 4% at birth and this level remained constant during the first postnatal week. The metabolism of 5-HT on the other hand remained relatively stable up to first postnatal day when 43% of infused serotonin was metabolized whereas on the 7th day after birth it was decreased to about 30%.

The results demonstrate the prostaglandin and amine insensitive ion develops differently during fetal and neonatal period in rabbit lung. Compared to our earlier result on the development of lung lipoprotein lipase in perfused rabbit lung (Kihno, Sjöberg & Martiala 1979) it is interesting to note that PKC ϵ metabolism and lipolysis were increased with age. However, lipolysis expressed peak lipidolysis preceding birth not seen with PKC ϵ . Consequently it is obvious that pulmonary metabolism is regulated by variety of factors in fetal and neonatal periods.

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Effect of cholera toxin on serotonin content of enterochromaffin cells in the cat small intestine

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Cholera toxin (CT) instilled in the small intestine causes massive secretion. In accordance with the hypothesis that serotonin (5-HT) may be the diarrhoeagenic messenger, it has been proposed that 5-HT may be involved in the secretory response of the intestine to CT (Fujita et al 1974). Since most of the intestinal 5-HT is localized to the enterochromaffin cell (EC), the effect of CT on the 5-HT content of EC in the cat small intestine was studied.

The experiments were performed on adult chloroform-anesthetized cats with bilaterally denervated jejunum. Following preperitoneal splanchnic denervation, segments of the jejunum were isolated. Crude extract of CT or heat inactivated CT was instilled in the segments for 1/2 h and then removed. Intestinal fluid transport was continuously recorded. After 1 to 5 h, specimens of the segments were removed, frozen in liquid propane, freeze-dried and treated according to the HPLC procedure. Sections of the specimens were studied by fluorescence microscopy and the fluorescence intensities of individual EC in coded sections were measured with a LKB 1111 cytofluorimeter. From each specimen the fluorescence intensity of 20 randomly selected EC were recorded (cf Ahlman 1976; Pettersson 1979).

In cats where a typical secretory response to CT was observed, the cytofluorimetric measurements revealed a significant ($p < 0.005$) decrease by about 70% in the fluorescence intensities of EC. CT-exposed segments as compared to 0 h control segments. In 6 h control segments the decrease was about 25-30%. The CT induced decrease in 5-HT fluorescence was observed in EC located both in the crypts and on the intestinal villi. The magnitude of the decrease was greater than that observed (about 30%) after vagal or splanchnic nerve stimulation for 10-60 min (Ahlman 1976; Larsson et al 1979). In cats where no typical secretory response, but rather absorption was observed despite the CT installation, the 5-HT fluorescence of the EC did not differ significantly from 5 h control segments. Those observed in cats exposed to heat inactivated CT.

These results demonstrate that the physiological response to CT is well correlated to a marked decrease in 5-HT content in intestinal EC, and support the hypothesis that 5-HT may be involved in the secretory response to CT.

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Ca^{2+} -sequestration by microvesicles isolated from bovine neurohypophysis

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Nerve endings from the neurohypophysis contain microvesicles but their function has not been established. In the present study purified microvesicles with and without coats were isolated from bovine neurohypophyses using the procedure of Blitt et al 1977. The purified microvesicles were found to contain Ca^{2+} dependent ATPase activity and to be able to accumulate Ca^{2+} in the presence of ATP and Mg^{2+} . The purified microvesicles were similar to brain microvesicles as judged by electron microscopy and SDS-electrophoresis in polyacrylamide gels. As judged by SDS polyacrylamide gel electrophoresis their protein composition was very different from neurosecretory granules. In teased apart brain the microvesicles was a background of irregular patches and lattice of hexagons and pentagons compatible with the structure of isolated coats.

The microvesicles contained Ca^{2+} -dependent ATPase activity. The activation by Ca^{2+} of the enzyme could be seen at Ca^{2+} concentrations down to 10^{-7} M free Ca^{2+} (in the presence of 2 mM Mg^{2+} and 1 mM ATP). The specific ATPase activity was 20 $\mu\text{mol}/\text{mg}$ protein per h. Possible ATP hydrolysis by contaminating Na^{+} K^{+} ATPase activity is not likely

since ouabain was added to the medium.

In the presence of ATP and Mg^{2+} the microvesicles accumulated Ca^{2+} . Addition of the ATP analogue AMP P-C P instead of ATP caused no Ca accumulation. Oxalate (10 mM) stimulated Ca^{2+} accumulation and the Ca^{2+} ionophore A23187 inhibited the accumulation. Under optimal conditions Ca^{2+} uptake was 340 nmol/mg of protein (steady state) in the presence of oxalate. The present data show that microvesicles similar to those prepared from brain can be isolated from the neurohypophysis. The microvesicles may have an important function in calcium sequestration. The data from SDS polyacrylamide gel electrophoresis support the hypothesis that these microvesicles are not retrieved membrane from neurosecretory granules that have emptied their contents.

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C 18

Calmodulin and ATP dependent accumulation and transport in subcellular fractions from the ox-neurohypophysis

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In the neurohypophysis calcium ions (Ca^{2+}) mediate secretion of vasopressin and oxytocin (review see Thorn et al 1978). To maintain sensitivity cytosolic free Ca^{2+} is held low at rest (pCa 8.7) by protein buffering and ATP-dependent sequestration into intracellular organelles as well as expulsion across the plasma membrane. We have studied the effect of the calmodulin dependent modulator protein calmodulin (reviewed recently by Chung 1980) on ATP-dependent $^{45}\text{Ca}^{2+}$ uptake into subcellular fractions from the ox-neurohypophysis. In microsomal fraction (Rusell & Thorn 1975) pretreated with EGTA (10^{-3} mol/l) to remove endogenous modulator calmodulin maximally elevated $^{45}\text{Ca}^{2+}$ accumulation four fold at an incubation pCa of 7.8 in a medium (pH 7.0) containing (in mM): 130 KCl, 20 MgCl_2 , 2 Mg_2ATP , 2 $^{45}\text{Ca}^{2+}$, 0.05 CaCl_2 , 0.2 EGTA, 0.5 $^{30}\text{O}_2$. The amount of calmodulin half-maximal for the effect was about 10 $\mu\text{g}/500$ μg membrane protein. In this membrane fraction ATP-dependent Ca^{2+} uptake and passive efflux are sensitive to cAMP. We are extending this study to include more clearly defined membrane fractions.

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Synaptic activity in lumbar spinal motoneurons of embryonic chick

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Fig. 1 Intracellular record from GL motoneuron to graded stimulation of the GL nerve. Averaged sweeps. Chick 21 days.

Chick has been used extensively in anatomical studies of neural development. We have now begun to study the development of synaptic activity in lumbar spinal motoneurons during the last 48 hours hatching using intracellular recording.

Chick aged 19-21 days were decapitated and perfused with 4-tubocurarine. The lumbar region of the spinal cord (segments 25-30) was exposed through a window in the shell. Various

branches of the sciatic nerve were cut peripherally and isolated. Intracellular recordings of synaptic activity were made using standard techniques. Microelectrodes had resistances of 30-40 Ω . Recordings could be made in which the resting membrane potential (60-70 mV) and action potential (ca. 100 V) remained stable for up to 2-3 h or more. Motoneurons innervating the lateral gastrocnemius (GL) muscle were identified by activating the cell body antidromically on stimulation of the sciatic nerve. In addition to the antidromic action potential GL motoneurons responded to graded stimulation of the GL nerve with graded early (6-8 msec) EPSP. The fibular (tibial and sural) nerves gave only later, usually more variable and predominantly inhibitory synaptic effects (Fig. 1). The homonymous early EPSP was often elicited by stimulus intensities subthreshold for antidromic activation of the motoneuron and was usually maximal at three times the intensity required to elicit the motor axon. The EPSP was altered in amplitude by depolarizing current injected into the cell but was unaffected by hyperpolarizing current. It fired on repetitive (50 Hz) stimulation caused depression of the amplitude of the EPSP.

We suggest that the early EPSP of GL motoneurons in the chick is mediated by chemical synapses and is analogous to the homonymous monosynaptic EPSP obtained in mammalian motoneurons in response to stimulation of low-threshold motor afferents.

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C 20

LOCATION OF MEMBRANIC SYNAPTIC POTENTIALS IN HIPPOCAMPAL PYRAMIDAL CELLS

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Low-threshold opening responses may act as a signal for neighbouring synapses (Burke 1967). We wanted to see whether synaptic information can be transmitted from a pyramidal cell.

In transverse hippocampal slice preparations, low-threshold selective activation of afferent input fibres. The pyramidal cell lies with its apical and basal dendrites parallel to the surface of the slice. Different fibres also run parallel to the slice surface but perpendicularly to the dendritic axis. Cuts along the dendritic axis may be used to restrict the afferent input to small and identifiable parts of the dendritic tree. The present study lesions restricted the afferent input to two or three fibres lying at different distances from the cell body.

In a pyramidal cell we activated orthodromically with subthreshold current so that pure EPSPs are evoked. A stimulus sequence consisting of three different stimuli. First, stimulation of the afferents running through the proximal dendrite, second the distal dendrite and third both together. The EPSPs from

number of such stimuli sequences were averaged for each cell and for the double input. The result from the individual stimuli was then compared to the algebraic sum of the EPSPs from the activation through each input alone.

When the cell was kept at its resting potential the algebraic sum was more prolonged in 65% of the cells than the EPSP due to the double input. When the cell was hyperpolarized however the EPSP resulting from the two types of stimulation were identical suggesting linear summation of the EPSP due to the proximal and distal inputs. The non-linear summation of the depolarized membrane potential was probably due to an interference by the IPSP whose effect was masked by hyperpolarization. The linear summation of the hyperpolarized membrane potential was seen in 15 of 16 cells.

In conclusion linear summation of EPSPs from neighbouring excitatory synapses occur at a hyperpolarized membrane potential. This might indicate that the synapses do not interfere with each other. However, definite conclusions must await experiments where the IPSP is pharmacologically blocked.

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Pharmacological effects on sleep in the immature animal

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In studies on adult cats now indicate that divergence in either direction from an optimal level of noradrenergic (NA) activation inhibits paradoxical sleep (PS) (Putkonen 1979), and as the balance between NA and 5-HT brain stem systems seems to be crucial in sleep regulation we have studied the effect of pharmacological manipulation of the NA 5-HT and acetylcholine systems on sleep in kittens. In these, quiet sleep (QS) is virtually absent at birth, and monoaminergic regulation is claimed to have very little role during the first postnatal weeks (Adrien 1978).

We have made day-time polygraphic recordings from kittens between 6 days and 2 months postnatal age during 8 hrs after i.p. drug injection with the kitten separated from its mother.

Most effects were in adult cats already from 1 week of age. While clonidine (CLO) 10 µg/kg inhibited PS, phentolamine (PHE) from 5 mg/kg increased PS by increasing episode duration and the probability of PS directly after waking. PHE 20 mg/kg decreased QS. At 1 month the larger dose was less effective.

although it still antagonized CLO. Yohimbine (YOH) increased arousal and state transitions and 2 mg/kg antagonized CLO. The postsynaptic blocker prazosin (PRZ) 1 mg/kg increased PS episode durations but lowered the PS incidence. At 3 weeks maximal PRZ effect was obtained with 5 mg/kg, but from 1 month with 1 mg/kg. PRZ always potentiated the effect of CLO.

Gitaloprin (GIT) 5 mg/kg, which inhibits 5-HT uptake, inhibited PS already at 1 week as in adults. In PCA treated animals GIT tended to normalise sleep.

Atropine (ATR) 5 mg/kg inhibited PS and increased QS from 1 week of age. ATR 0.075 mg/kg slightly shortened PS episodes but in combination with PRZ (20 mg/kg) inhibited PS radically.

The results show that sleep regulation in kittens depends on similar systems as in adults although the characteristics of QS are not fully developed. The differences are mainly quantitative and the sensitivity to the studied agents changes during development.

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Metabolic consequences of membrane depolarisation

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In excitable tissues membrane depolarisation is mainly an electrical event and the depolarisation in itself initiates the next depolarisation and by this the propagation of the impulse.

However a series of non-excitable tissues also depolarise when they are stimulated. These tissues include brown adipose tissue and it has been found that the resting membrane potential of 80 mV is diminished to 25 mV when the tissue is stimulated by norepinephrine and thermogenesis is activated. The consequences and function of this depolarisation have however remained unknown.

We have recently demonstrated (Al Shaikhly Nedergaard & Cannon 1979) that brown fat mitochondria possess a mechanism for Na⁺ stimulated Ca²⁺ release.

We have now found that this mechanism allows the mitochondria to regulate the extramitochondrial (in the cell) steady state Ca²⁺ concentration accurately in the range 0.3-1 µM dependent on the cytosolic Na⁺ concentration. This steady state Ca²⁺ level is

most sensitive to changes in Na⁺ concentrations in the range 5-15 mM.

We have also found that it is possible by this mechanism to regulate the activity of the mitochondrial glycerol 3-phosphate dehydrogenase (which is Ca²⁺-dependent). This enzyme plays a key role in regulating cytosolic concentrations of glycerol 3-phosphate and an increased activity of the enzyme will help channel activated fatty acids towards combustion instead of towards (re-)esterification.

Based on these experiments and on the sensitivity of norepinephrine stimulated respiration (but not lipolysis) to extracellular Na⁺ we suggest that the metabolic significance of membrane depolarisation in non-excitable tissues may be related to a Na⁺-dependent increase in cytosolic Ca²⁺ with regulatory functions as a result; in brown fat these may involve the control of the reesterification process.

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influence of LH and FSH on the adenylate cyclase activity in granulosa cell isolated from preovulatory follicles

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Preovulatory granulosa cells isolated from the ovaries of HMG treated immature rat responded to FSH as well as LH in vitro with increased cAMP formation, although FSH was more effective (Hansson et al, 1978). The aim of the present study was to explore whether this stimulation of cAMP formation was correlated to an enhanced adenylate cyclase activity in these cells.

Preovulatory granulosa cells were isolated as described by Hörstenström et al (1980). Preparation of membranes and the assay for adenylate cyclase activity was performed with some modifications.

Described by Khan and Rosberg (1979). Each sample contained membranes from granulosa cells from 8-10 follicles.

The adenylate cyclase activity was significantly stimulated by low concentration (0.01 μ M) of either FSH or LH. A constant ratio of 1 μ g/ml of FSH and 50 μ g/ml of LH was needed to elicit maximal response. When GTP analogue (Guanylyl-imidodiphosphate) was added to the adenylate cyclase assay there was 10-fold increase in the enzyme activity although the relative stimulation of FSH and LH was not altered.

These results are in good accordance with the findings that both LH and FSH in vivo stimulate cAMP formation in isolated preovulatory granulosa cells. This also indicates that the increased cAMP

formation depends on an effect of the gonadotropins on the receptor-adenylate cyclase level in the granulosa cell membrane. Furthermore it suggests higher sensitivity of these cells to FSH as compared to LH.

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C 24

Increased receptor binding affinity of 125 I-glucagon when Met²⁷ is oxidized during the iodination

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Glucagon is widely used in radioimmunoassay and biological work as tracer for the 29 amino acid polypeptide hormone glucagon. Until recently we iodinated glucagon using the chloramine-T method (CHL-T). However, HPLC chromatography revealed that the ligand of Met²⁷ was oxidized. This oxidation did not occur when glucagon was iodinated using the lactoperoxidase method (LPO). Oxidation (Markussen & Larsen 1980).

Glucagon was iodinated by CHL-T (Jørgensen & Larsen 1972) (specific activity 319 Ci/g) by LPO (Thorell & Johansson 1971) (specific activity 322 Ci/g). The specific binding (defined: total binding (tracer only) minus unspecific binding (trace plus uptake of unlabelled glucagon)) of these two trace preparations to isolated adipocytes and hepatocytes measured as described (Gliemann & Sonne 1978; Sonne et al 1978). When the concentration of trace 1 K_d for the binding of glucagon to the cell bound hormone/f as hormone (B/F) became identical with the dissociation constant of receptors (K_D /dissociation constant (K_d) of the labelled ligand. There-

fore when carried out the same cell pool (i.e. same K_D) variations in B/F reflect variations in K_d .

Only half as much 125 I-glucagon labelled by CHL-T was bound compared to the trace labelled by the LPO method (Tabl. I) indicating that the C terminal part of the glucagon molecule is of importance for the interaction with its receptor. Similar results were obtained on two other batches of tracers.

Tabl. I Binding affinity of 125 I-glucagon labelled by the CHL-T or the LPO method. 125 I-glucagon (10 pM) was incubated 37°C for 30 min with 1.8 (v/v) adipocytes or for 60 min with hepatocytes (5x10⁴ cells/ml). B/F indicates the value for the specific binding. Mean of 3 replicates.

| 125 I-glucagon | Adipocytes B/F 10 ³ | Hepatocytes B/Fx10 ³ |
|---------------------|-----------------------------------|------------------------------------|
| LPO | 4.4 | 2.2 |
| CHL-T | 2.5 | 1.1 |

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Ipsi and contralateral cold sensitivity after unilateral lesions of the lateral spinal funiculus of the cat

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Behavioural thermosensitivity of the paws was investigated before and after unilateral surgical lesions of the lateral spinal funiculus above the 5th cervical segment. The sensitivity for skin temperature decreases was investigated independently for either body half on alternate days in an automatic T-maze. The cats' performance of the discriminatory task with either body half could be sustained whenever necessary by an acoustic cue which was delivered optionally in unison with the thermal cue.

One cat received two subsequent lesions of the same lateral funiculus (1 = partial transection involving ventral half, 2 = complete transection). In two more cats the whole of one lateral funiculus was transected immediately. No lesion caused any change of the ipsilateral sensitivity. The ipsilateral paws were used to discriminate equally small temperature decreases pre- and postoperatively if $\pm 4^\circ\text{C}$ or less. Neither was any contralateral change found after the first partial lateral funiculus lesion of the cat who was operated two times.

The complete lateral funiculus transections caused profound contralateral thermosensory disturbances in all instances. There was a residual sensitivity however which in the best case consisted of an eventual ability to discriminate a temperature

difference of 20° continuously above criterion level (22/32 correct responses/session) for a long period. This lowered thermosensory capability perhaps was due to something else than a simple sensory attenuation because there was a lack of interaction between the body halves. The above cat who was performing a coarse discrimination with the defective side was performing a much more difficult task with the other side on alternate days. The performance on the defective side failed to improve for its simple task, however, when the task on the other side was made even more difficult and demanded an increasingly larger behavioural effort.

It was found previously (NorrSELL 1979) that unilateral lesions of the lateral funiculus caused contralateral thermosensory defects in the cat but the technique did not permit quantitative estimates. Now it may be concluded that unilateral interruptions of the lateral funiculus cause drastic and purely contralateral defects of the cold sensitivity. The defective side retains a residual thermosensitivity by having the means of being useful for discrimination of large temperature differences but may lack normal thermal sensations. The findings are most easily explained by supposing that the lesions interrupted a crossed ascending thermosensory pathway which is necessary for normal behavioural thermosensitivity.

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C 26

Binocular activation of perigeniculate neurones in aquint operated cats

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The perigeniculate cells serve as interneurons in a recurrent inhibitory pathway to principal cells in the lateral geniculate nucleus. Most perigeniculate neurones receive excitation from both eyes through collateral connections from monocularly activated principal cells. We have now studied the binocular input to perigeniculate neurones in aquint operated cats. It is well known that such an operation at an early age leads to a disruption of normal binocular connections from principal cells to neurones in the primary visual cortex.

Three kittens were made aquint by sectioning of the left lateral (two kittens) or medial (one kitten) rectus muscle 21 days after birth. The animals were studied about two years later in acute experiments. We first recorded from 30-40 neurones in the primary visual cortex of each animal. As expected for aquint operated cats most cortical neurones were activated from either the left or the right eye. Only 14% of the cells were found to have a binocular input.

The perigeniculate neurones in the same animals seem to receive a completely normal binocular input. Of 60 identified cells 78% could be activated via both eyes as compared to 73% in normal control animals. The distribution of eye preference was also the same in the aquint operated cats as in the normal.

The visual cortex (areas 17 and 18) was removed during the experiment in one aquint operated cat. Binocularly activated perigeniculate cells were found also after this lesion, indicating that the excitation was mediated in subcortical connections as in normal animals. Thus perigeniculate neurones seem to be able to maintain binocular connections from principal cells despite of a non-congruent visual input, while cortical neurones lose binocular connections in this situation. This difference in plasticity at the two termination sites for the principal cells suggests that postnatal reorganization of synaptic connections may be functionally specific and controlled by the postsynaptic neurone.

DEMONSTRATIONS

Demonstration of a technique for evaluating recurrent inhibition in man with on-line computer analysis

H HULTBORN R MACKEL & H WIGSTRÖM

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A method that is based on paired Hoffman reflexes has recently been developed to estimate recurrent inhibition in man (Bossel & Pierrot Desalligny 1977). This technique has later been used to deduce the supraspinal control of Renshaw cells during various ankle movements (Hultborn & Pierrot-Desalligny 1979). The data obtained are subjected to on-line analysis by a digital computer (Nord-10/8) to provide the necessary guidance for the following steps in the experiment. Peak to-peak amplitudes of the evoked EMG signals are measured within several adjustable time windows and the results are displayed in diagrams on a graphic screen. Triggering of the stimulating sequence is controlled by the computer which is useful for instance in the analysis of voluntary tracking movements.

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Evidence that the non-reciprocal inhibition of spinal motoneurons by Ia muscle spindle afferents and by Ib tendon organ afferents is mediated by the same interneurons

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Recent observations have shown that impulses in Ia muscle spindle afferents may cause inhibition of motoneurons of synergist as well as of antagonist muscles (Feiz et al 1979 Jankowska et al 1980). They showed also that the pattern of distribution of the Ia non-reciprocal inhibition closely resembles the pattern of Ib inhibition (Jankowska et al 1980). The possibility has now been considered that the same interneurons mediate the Ia non-reciprocal and Ib inhibitory actions and in order to test this possibility the following experiments were performed. DI and triaxial IPSPs evoked by Ia and Ib afferents of triceps surae were recorded in a number of motoneurons of plantaris flexor digitorum longus and quadriceps muscles. Ia afferents were activated by adequate stimuli (brief triangular stretches of 10-20 mm which were below threshold for Ib afferents of Feiz et al 1979). Ib afferents were excited by electrical stimulation of the nerves to the same muscles using technique of Coppin et al (1970). The technique consists in raising the threshold to electrical stimuli for Ia afferents above that for Ib afferents by a prolonged vibration of the muscles and allows selective activation of a certain proportion of Ib afferents. A comparison was then made of IPSPs evoked by Ia and Ib afferents stimulated separately and by properly timed stimulation

D 2

of both of them. Spatial facilitation of effects of Ia and Ib afferents has been disclosed in a great proportion of the analysed motoneurons indicating their mediation by common interneurons (cf Lundberg 1979). Our results show thus the existence of a shared pathway of Ia non-reciprocal and Ib inhibition although they do not preclude the possibility that inhibitory actions of muscle spindles and tendon organs are evoked via both common and separate pathways.

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phases of the non-adrenergic non-cholinergic innervation of the stomach of the rainbow trout *Salmo gairdneri* using ¹⁴omega preparation

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The vagal parasympathetic innervation of the stomach of the rainbow trout is inhibitory causing relaxation of the stomach followed by "rebound" contraction shortly after the end of stimulation (Campbell 1975)

In present experiments are performed on mice perfused stomach measuring the intramural pressure as well as the perfuse on flow by perfusing perfusion fluid directly used to perfuse an isolated strip of gastric smooth muscle, mounted in petraffin oil (see f. g. 1975)

Stimulation of the vagus nerve to the whole perfused stomach produces small inhibition of the intramural pressure during the stimulation followed by large "rebound" contraction of the stomach and simultaneous reduction in flow rate to 50% after cessation of the stimulation

The identified substance (transmitter) released in the perfused stomach by nerve stimulation suppresses the isolated strip producing the same response of the strip as initial small inhibition (see inhibition of spontaneous activity) followed by pronounced contraction. The effect can be blocked by direct transmural stimulation of the isolated strip. The response to transmural stimulation can be blocked by tetrodotoxin indicating an effect is nerve mediated release of transmitter

The responses of both whole stomach and strip are not blocked by tropine hyoscine phenolamine or propranolol indicating the presence of non-adrenergic non-cholinergic type of innervation of the stomach of the rainbow trout (see also Groves & Campbell 1979)

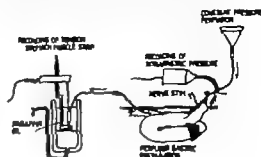


Figure The ¹⁴omega preparation; perfused whole stomach and an isolated stomach muscle strip perfused by the outflow from the whole stomach

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D 4

EFFECTS OF HYPOXIA AND HYPERCAPNIA ON CENTRAL NERVOUS SYSTEM (CNS) NEURONS AND ON PERIPHERAL SYMPATHETIC ACTIVITY
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Previous biochemical studies have shown that both hypoxia and hypercapnia can markedly affect brain monoamine metabolism. The physiological significance of these changes is, however, difficult to evaluate based on biochemical data. It has been suggested by Cohen and coworkers that hypoxia causes activation of brain NE neurons. The brain NE neurons in the nucleus locus coeruleus (LC) seem to be involved in many of behavioural (wakefulness, anxiety, stress) and physiological (cardiovascular) functions. Thus, we have utilized single unit recording techniques to study directly these brain NE neurons under hypoxic hypercapnic as well as 100% O₂ in the inspired air. In the same experiments peripheral sympathetic nerve activity was registered in order to clarify the possible connection between central and peripheral NE neurons.

Hypercapnia (2-15% CO₂ in the inspired gas mixture) caused simultaneous increase in both LC neuronal firing rate and sympathetic nerve activity. During hypoxia (about 10% O₂ in the inspired gas mixture) the LC neuronal firing rate was increased while the sympathetic nerve activity was decreased. When the pO₂-level was increased by letting the rats breathe 100% O₂ the LC neuronal firing rate was markedly reduced, while the sympathetic activity if any-

thing, was increased.

It is concluded that alterations in the availability of oxygen can markedly affect not only brain monoamine metabolism but also the physiological activity of the NE neurons. The data indicate that whereas in several situations central and peripheral NE neuronal activity is simultaneously and qualitatively similarly affected, this does not appear to be general principle.

This work was supported by the Swedish Medical Research Council (projects nos 470, 4764 and 00016)

A longla tng afterhyperpolarization following single spikes in CA1 pyramidal cells in the hippocampus
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In most central neurones studied the soma-dendritic spike is followed by a longlasting afterhyperpolarization (AHP). This AHP is caused by a calcium activated permeability increase to potassium ions and is a major intrinsic factor for firing control. In contrast to most other cells hippocampal pyramidal cells are believed to lack this AHP the spike being followed only by a longlasting afterdepolarization (ADP) (Kandel & Spencer 1961). However it was recently shown that longlasting repetitive activation of CA1 pyramidal neurones was followed by a longlasting AHP (0.5-1 sec) probably caused by a calcium-activated potassium conductance (Holtson Schwartzkroin & Prince 1977). The question then arising is whether this AHP can also be detected following single or short train of spikes. In the present experiments (performed on transverse slices of guinea pig hippocampus) CA1 pyramidal neurones showed an AHP following a single or a short train (2-5) of spikes. This AHP which followed the ADP generally consisted of two phases: an initial one of shorter duration (200-350 msec) reaching a peak amplitude 50-125 msec following spike onset and a later one of long duration (2-5 sec) reaching a peak amplitude some 500 msec following spike onset. Following a single spike the AHP was usually dominated by its initial phase (peak amplitude around 1 mV) the later phase could be absent. However with repetitive activation the later component

grew generally more than the initial one and the AHP could be dominated by its later phase. The AHP was associated with a change in conductance the time course of which ran parallel to that of the AHP. Thus when the AHP was dominated by its later phase the conductance change increased slowly with time reaching a maximum value some 500 msec following spike onset. Following the peak of the AHP the conductance change showed an exponential decay. The AHP amplitude was potential-dependent decreasing and reversing in polarity with hyperpolarization of the membrane. The reversal was however asymmetrical the initial phase reversing to a more hyperpolarized level. Thus as in other central neurones the spike in CA1 pyramidal neurones is followed by an AHP presumably caused by a potassium conductance increase. Compared to other neurones this AHP is unusually longlasting with a duration up to 5 sec which is 50-100 times longer than that normally found. Some findings indicate that the AHP can be composed of two somewhat different ionic processes.

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D 6

Chang th t f amy l se ec tion d ng
p l g d t mul n on

B ASKING P GJÖRSTRUP & H PÄRSSON

1 tit t f Phy l l gy Unive lty f Lund
Svede

In xpe lme t on l f ry amy l ecretion l f l
gene lly used that th magntud f the ecr-
et ry pon l d pend t on the amou t f p r-
f reed amy l se l the gland T bt l unlf r p r-
exp lment l condition the nme l ually
t reed f 24 hou l t that time b t 90% f
th amy l l found l the ec t ry gram l
f t ion (P tten Will am & Cope 1977)
Stimul tions sp lment then ft carried
out f vo l bou b t c l t known l he
t f amy l ecretion d g cont l ou de-
pl t ion l ff t d by th de d amou t f
amy l l the gland l t known l only the
pref reed amy l sva l bl f se t ion
In bblt unda than the f th lght
pa tduct wa ca l t d t ll t lva l
5 ml l s val l xpe lment l tng 2 hou
The amy l se t ion vok d by sympathe t l
mul t ion at l ks va rel ed l l ry flow
(60-80 l/ml) p oduced by p r sympathe t rva
c lmul t ion t 1.5-2 H (GJB tryp 1979)
The t lmul t ion were continuous th oughout the
experiment
Th reat lnd l t th t d l g th fl t 25 20
ml f t lmul t ion the amy l se once t t ion l
about 3000 unit / l but ft 60 ml lth h a d
c e ed t 25 50% lthough at l ast 50% f the
gland lar content f amy l remained. An the
60 ml of t lmul t ion f rther de sed the s ll

ry con e t ion t bout 10% f l l t l l valoe
In th xperlme t abbt p otid l o l were
lncub ted l g d Kr ba-Rl gr med l f 2
ho and t lmul t d with l op on ll 10⁻⁶ M
d r l g th f r t h ou (Askng & GJB tryp 1980)
Th t l se wa he hed and re l cubat d f
smothe hou with Kr b R nger upplemeted lth
amino l d g t Egl MED
At the end f th l p lod th amou t f amy l
e m l ng l the t uo ad that rel d l t
b th wa say d t obt l the t l l amou t
In fou xpe lme t the tot l amou t f amy l l
un t lmul ted ontrol and in th t lmul ted t l ou
found e be th am amy l g between
1300-1500 un e /100mg t l se l control 90% f
the amou t wa found the t l uo well 50% wa
l ft l the t mul ted t l uo
Th pe me t suggest that lthough l rge amou t
f amy l may em sv l bl f cra l on the
t f amy l l ca not be ml t l oed but
d w th lnc l g depl t l f amy l se
Th re l t lso gge e that only p r reed xpe-
l l l t ed whlch l support d by th l k l
amy lase f r t ion l the l t pe lme t

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Er-ergocryptine (CB-154) inhibits prolactin (PRL) and growth hormone (GH) production in cultured pituitary tumour cells

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pituitary tumour cells (GH₃ cells) which secrete and secrete PRL and GH *in vitro* react to several physiologically occurring antiproliferative agents. Theophylline (THEO) and 17 β -oestradiol (E₂) are similar analogues to normal pituitary cells. Short-term dopamine (DA) treatment inhibits PRL secretion in these cells (1). We have studied the effects of the ergot derivatives CB-154, which acts as a potent dopamine receptor agonist in the anterior pituitary gland (2) and the leading α -dihydroergocryptine to GH₃ cells at 37°C. In concentrations of 10⁻⁶ M, CB-154 inhibited PRL secretion by about 30% after 30 min and synthesis by about 60% after 3 days of treatment. GH secretion and synthesis as well as cell growth were not significantly affected. This secretion. However, raising the concentration of CB-154 (above 10⁻⁶ M) an increasing inhibition of PRL synthesis (more than 90%) was observed in addition to reduction in GH pro-

duction and cell proliferation. These effects were reversible on cessation of CB-154 treatment. Intracellular electrophysiological registrations showed inhibition of spontaneously occurring action potentials in the GH₃ cell. After 6 days of treatment the cell proliferation was reduced about 80% in comparison with controls. Microflow cytometry demonstrated an inhibition in cell cycle traverse in the G₁ phase of growth. 3. Binding of α -dihydroergocryptine was rapid, saturable and partly reversible at 37°C. In conclusion, CB-154 has an effect analogous to DA in GH₃ cells by inhibiting PRL and GH secretion and synthesis in a dose and time dependent manner. The short time effects occur rapidly and cause changes in cell membrane characteristics and hormone secretion while longer treatment periods cause inhibition also of hormone synthesis and cell growth.

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D 8

Capillary supply and cross sectional area of low and fast twitch fibres in skeletal muscles from mice and females.

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From skeletal muscle the degree of capillarization of each of the major fibre types is difficult to evaluate separately as the low and fast twitch fibres are heterogeneously mixed and capillaries are shared between different fibre types. One approach to the problem is to analyse muscles with large differences in fibre composition. In mice healthy young subjects (6 men and 6 women, sedentary to well trained) had muscle biopsies taken from triceps brachii (TB) vastus lateralis (VL) and soleus (S) fibres. Isotopic analysis of low twitch (LT) and fast twitch (FT) fibres was based on histochemical staining for myofibrillar ATPase after preincubation at pH 4.3 and 10.3. Capillaries were histochemically visualized with the myelase-PAS method. Cross sectional fibre area and the mean fibre diameter were determined from photographs of the myelase-PAS stain. The fibre composition was for the 39% ST for 11.6% ST and for 5.7% ST with no differences between the sexes. The number of capillaries around each FT fibre was less in TB compared to VL or S. In men and women (Table 1) (p < 0.05). For the FT fibres CA was less in TB than in (p < 0.05). Part of the explanation for the variation in CA was the difference in cross sectional area of the fibre (Table 1). For FT fibres men had larger FA in TB and VL but not in S (p < 0.05). Comparing ST

fibre between the three muscles the FA was smallest in TB compared to VL or S for men and women (p < 0.05) with similar trend for FT fibres. To evaluate capillary density for the two fibre types the variable fibre type area per capillary (AC) was calculated. This variable was significantly different between ST and FT fibres in all the muscles for the men and in TB for the women.

Table 1. Mean values for capillaries around (CA) slow and fast twitch (ST, FT) fibres. Their cross sectional area (FA) supplied by each capillary (AC) in triceps brachii (TB) vastus lateralis (VL) and soleus (S).

| | | TB | | | VL | | | |
|---|----|-----|-----|-----|-----|-----|-----|--|
| | | CA | FA | AC | CA | FA | AC | |
| CA (no) | ST | 9 | 3.9 | 0.9 | 4.6 | 6.9 | 7.6 | |
| | FT | 5.1 | 3.7 | 6.2 | 4.0 | 6.6 | 8 | |
| FA (μm ² × 10 ³) | ST | 3.9 | 2.9 | 2 | 5.0 | 6 | 7.8 | |
| | FT | 3.8 | 3.9 | 4 | 4.4 | 8.8 | 7.9 | |
| AC (μm ² × 10 ³) | ST | 0.8 | 0.8 | 0.8 | 1.0 | 0.9 | 1 | |
| | FT | 1.1 | 1.0 | 0 | 1 | 1.3 | 0 | |

Several conclusions are possible. First, there are differences in the areas supplied by each capillary when comparing ST and FT fibres. Second, that the differences which exist in CA between the muscles are likely related to the size of the fibres and third, that there are also sex differences in those variables measured.

β -ergocryptine (CB-154) inhibits prolactin (PRL) release from rat pituitary (GH) production in cultured rat pituitary tumor cells

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 College of Veterinary Medicine Oslo and
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Pituitary tumor cells (GH₃ cells) which synthesize and secrete PRL and GH *in vitro* react to several physiol. logically occurring neurohormones as thyrotrophin (TSH) and 17 β -oestradiol in a manner analogous to normal pituitary cells. Short-term depolarization (DB) treatment inhibits PRL secretion in these cells (1). We have studied the effects of the ergot derivatives CB-154, which acts as a potent dopamine receptor agonist, is the gate for pituitary gland (2) and the binding of 3 H-dihydroergocryptine to GH₃ cells at 37°C.

Low concentrations of β -ergocryptine (5a10⁻⁶M) inhibit PRL secretion by about 30% after 30 min and synthesis by about 60% after 3 days of treatment. GH secretion and synthesis as well as cell growth were not significantly affected. This concentration however failing the concentration of CB-154 (above 10⁻⁶M) an increasing inhibition of PRL synthesis (more than 90%) was observed in addition to a reduction in GH production and cell proliferation. These effects were reversible on cessation of CB-154 treatment. Intracellular electrophysiological registrations showed inhibition of spontaneously occurring action potentials in the GH₃ cell. After 6 days of treatment the cell proliferation was reduced about 80% in comparison with control. Microflow cytofluorometry demonstrated an inhibition of cell cycle traverse in the G₁ phase of growth.

binding of 3 H-dihydroergocryptine was rapid, saturable and partly reversible. 17°C treatment in conclusion CB-154 has an effect analogous to DB in GH₃ cell by inhibiting PRL and GH secretion and synthesis in a dose and time dependent manner. The short time effects occur rapidly and cause changes in cell membrane characteristics and hormone secretion. While longer treatment periods cause inhibition also of hormone synthesis and cell growth.

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D8

Capillary supply and cross sectional area of slow and fast twitch fibres in skeletal muscles from mice and humans

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In human skeletal muscle the degree of capillarization of each of the major fibre type is difficult to evaluate separately as the slow and fast twitch fibres are heterogeneously mixed and capillaries are shared between different fibres. One approach to the problem is to analyze muscles with large differences in fibre composition.

Twelve healthy young subjects (6 men and 6 women, age 20-30 years) had muscles biopsied from triceps brachii (TB), vastus lateralis (VL) and soleus (S). Three classification into slow twitch (ST) and fast twitch (FT) fibres was based on histochemical staining for myofibrillar ATPase after preincubation at pH 3 and pH 5. Capillaries were histochemically visualized with the enzyme-PAS method. Cross sectional fibre area and the number of capillaries were determined on photographs of the myo-PAS stain.

The fibre compositions were: 39% ST for VL, 40% ST for TB and 77% ST for S with no differences between the muscles. The number of capillaries around ST fibres was less in TB compared to VL or S in men and women (Table 1) (p < 0.05). For the FT fibres, VL was less in TB than in S (p < 0.05). Part of the explanation for the variation in capillary area is cross sectional area of the fibres (Table 1). For FT fibres men had larger F in TB and VL but not in S (p < 0.05). Comparing ST

fibres between the three muscles the FA was smallest in TB compared to VL or S for men and women (p < 0.05) with similar trend for FT fibres. To evaluate capillary density for the two fibre types the "capillary fibre type area per capillary" (AC_{FT}) was calculated. This variable was significantly different between ST and FT fibres in all the muscles for the men and in TB for the women.

Table 1. Mean values for capillaries around (CA) slow and fast twitch (ST, FT) fibres, their cross sectional area (FA) supplied by each capillary (AC) in triceps brachii (TB), vastus lateralis (VL) and soleus (S).

| | | TB | | VL | | S | |
|---|----|-----|-----|-----|-----|-----|-----|
| | | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ |
| CA (no) | ST | 4.9 | 3.9 | 5 | 4.6 | 6.9 | 7.8 |
| | FT | 8.1 | 3.7 | 6.2 | 4.0 | 6.6 | 6.8 |
| FA (μm ² × 10 ³) | ST | 3.9 | 2.9 | 3 | 5.0 | 6.4 | 8 |
| | FT | 5.8 | 3.9 | 6.4 | 4 | 8.8 | 7.9 |
| AC (μm ² × 10 ³) | ST | 0.8 | 0.8 | 0.8 | 1.0 | 0.9 | 1.0 |
| | FT | 1.1 | 1.0 | 0 | 1.4 | 1.3 | 1.0 |

Several conclusions are possible. First, there are differences in the areas supplied by each capillary when comparing ST and FT fibres. Second, the differences which exist in CA between the muscles are likely related to the size of the fibres and third, there are only minor sex differences in these variables measured.

A longlasting afterhyperpolarisation following single spikes in CA1 pyramidal cells in the hippocampus
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In most central neurones studied the soma-dendritic spike is followed by a longlasting afterhyperpolarization (AHP). This AHP is caused by a calcium-activated permeability increase to potassium ions and is a major intrinsic factor for firing control. In contrast to most other cells hippocampal pyramidal cells are believed to lack this AHP; the spike being followed only by a longlasting afterdepolarization (ADP) (Kandel & Spencer 1961). However it was recently shown that longlasting repetitive activation of CA1 pyramidal neurones was followed by a longlasting AHP (0.5-1 sec) probably caused by a calcium-activated potassium conductance (Häusser, Schwartzkroin & Prince 1977). The question then arising is whether this AHP can also be detected following single or short train of spikes. In the present experiments (performed on transverse slices of guinea pig hippocampus) CA1 pyramidal neurones showed an AHP following a single or a short train (2-6) of spikes. This AHP which followed the ADP generally consisted of two phases: an initial one of shorter duration (300-350 msec) reaching a peak amplitude 50-125 msec following spike onset and a later one of long duration (2-5 sec) reaching a peak amplitude some 500 msec following spike onset. Following single spike the AHP was usually dominated by its initial phase (peak amplitude around 1 mV); the later phase could be absent. However with repetitive activation, the later component

grew generally more than the initial one and the AHP could be dominated by its later phase. The AHP was associated with a change in conductance the time course of which ran parallel to that of the AHP. Thus when the AHP was dominated by its later phase the conductance change increased slowly with time reaching a maximum value some 500 msec following spike onset. Following the peak of the AHP the conductance change showed an exponential decay. The AHP amplitude was potential dependent, decreasing and reversing in polarity with hyperpolarization of the membrane. The reversal was however asymmetrical the initial phase reversing at a more hyperpolarized level.

Thus as in other central neurones the spike in CA1 pyramidal neurones is followed by an AHP presumably caused by a potassium conductance increase. Compared to other neurones this AHP is unusually longlasting with a duration up to 8 sec which is 40-100 times longer than that normally found. Some findings indicate that the AHP can be composed of two somewhat different ionic processes.

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D 6

Change in the rate of synthesis during
prolonged stimulation

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Runde

In experiments on salivary amylase secretion it was generally assumed that the magnitude of the secretion at any point is dependent on the amount of preformed amylase in the gland. To obtain uniform experimental conditions the animals usually received 24 hours of fasting that time about 90% of the amylase is used and the secretion is very small fraction (7 then Williams & Cope 1977). Stimulus on experiment the food carried out for several hours but it is known if the rate of amylase secretion during continuous deprivation is affected by the raised amount of amylase in the gland it is known if only the preformed amylase is available for secretion on laparotomy under urethane anaesthesia the right pancreas did not contain 11 times as much amylase as the left experiment 1 and 2 hours. The amylase secretion evoked by sympathetic stimulation 1 Hz was raised in the liver flow (60-80 1/ml) produced by peripheral sympathetic stimulation 1.5 Hz (CJ8 tryp 1979). The stimulation were continuous throughout the experiment.

The results indicate that during the first 20 minutes of stimulation the amygdala secretes about 3000 units/ml but after 60 minutes the discharge is about 25-50% although at least 50% of the glandular content of amygdala remained. Another 60 minutes of stimulation further decreased the li-

ry con t t on c bout 10X f il l i a l v a l u e
I t h e x p e i m e t a b i t p r i d l o b u e s w
i n c u b e d i g s e d K r b e r - K i n g m e d i a f 2
h o a n d t i m u l d w i t h i o p e n l i 10 % M
d i g t h f t h o w (A s k i n g a G j b s t r u p 1980)
T h e t i w e h e w e h a d a n d r e i u b e r t f o r
a n t h e b o u w i t h K r b e r - K i n g s u p p l e m e n t e d i t h
a m i n o i d c o r d i g t E a g l M E M
A t t h e a n d f b i p e i o d t h a m o u n t f a m y l a
e m a i n i g t h t i s u e a n d t h a t r e l a s s e d i t
b t h w a s a y d t b t i b e t a m o u n t
I f o u x p e i m e t t h e t t l a m o u n t f a m y l a i
u n t i m u l t d c o n t r o l a n d i t h t i m u l t e d t i s u
w f o u n d b e t h e a s s e r a n g e g b t w o o
1300-1500 m m t / 100 m g i u e i n c o n t r o l 90 % f
t b a m o u n t w a s f o u n d i t h e t i u e w h l 50 % f
l f t i t h e t m u l t e d t i o e
T h x p e i m e s u g g e t h a t t h o u g h i t r e a s s e s s
f a m y l a s m a y a s s e s s l b l f a s e t o n t h e
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d e c r a s e w t h i g d e p l i t i o n t a m y l a s
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Effect of exercise on lecithin-cholesterol
acyltransferase (LCAT) ac y i men

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Lecithin-cholesterol acyltransferase (LCAT) has
control role in lipoprotein metabolism. Its main
function is to produce cholesterol esters in high
density lipoprotein (HDL) particles (see Glomset
1974). There is no evidence that LCAT may have
role in the removal of cholesterol from a vessel
wall. Very little is known about the regulation
of the enzyme activity. The present study
examined the effect of training on serum LCAT activity.

A group of 28 men trained under control for 15
weeks at least three hours a week. The training
consisted mainly of running or skiing. During the
experimental control group of seven men retained
the same physical activity. At the beginning
of the period the groups did not differ in role
in the role of body weight, age or physical
fitness. The effect of training on plasma lipids
and lipoproteins. These subjects have been
reported on (Peltonen 1980). Serum
LCAT activity was measured during the training
period (15 training and 1 control subject
according to Albers (1978)).

Training caused significant ($P < 0.05$) in-
crease in physical fitness, while no change

occurred in control. In the training group LCAT
activity increased from 13.9 ± 2.2 nmol/l/h to
 31.1 ± 3.2 nmol/l/h ($P < 0.01$ paired t-test).
Whereas in control the activities remained the
same before but not after the training. LCAT
correlated positively with serum triglyceride
($P < 0.05$) and with posthepatic plasma hepatic
lipase ($P < 0.10$) in the combined groups.

The present results suggest an increasing ef-
fect of training on serum LCAT activity. Whether
this is direct influence of training on the
enzyme is not clear. Apolipoprotein A1, which is an
activator of LCAT, is also increased in physical-
ly active subjects (Lehtonen 1979) and
this could be one possible explanation for the
observed change. Together with the increased HDL
cholesterol concentration (Peltonen et al 1980)
the elevated enzyme activity could have bene-
ficial effect on cholesterol removal from the
vessel.

Supported by Yrjö Jahnsson Foundation, Finland

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D 12

Exercise induced changes in human serum fatty
acid composition

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The fatty acid composition of lipids in the blood
and different tissues has been found to be related to
various health surveys, especially to the
search for new risk factors of atherosclerosis
(see Byberg 1975, Sanders 1978).
The role of physical exercise has generally not
been taken into account as possible effector of
human fatty acid composition. In the present study
we have analyzed the serum total fatty acid com-
position during controlled physical training
program.

The duration of the training program (Peltonen
et al 1980) was 15 weeks consisting mainly of
running or skiing under control. After three
weeks the subjects (28) and the control
group were previously on raised healthy men.

The total lipid fraction of the fasting serum
samples was isolated and purified essentially
according to Folch (1957). The fatty acids
were analyzed as their methyl esters (van Wijk-
gaard 1967) by capillary gas chromatography
using internal standard in the liquid phase
and flame ionization detection.

During the test period there appeared to be
tendency to reduced level of polyunsaturated long-
chain fatty acids in serum. A significant de-

crease was found in the concentration of 22:5
($P < 0.05$) and 22:6 ($P < 0.02$) acids as compared to
the control. In 28 subjects and 20 subjects the small reduc-
tion (10-20%) was not however statistically sig-
nificant. When calculated as combined group the
long-chain (18:1 to 22:6) polyunsaturated fatty acids
decreased significantly (about 10% $P < 0.05$)
during training. The 16:0, 18:0, 18:1, 18:2, 18:3 acids in-
creased significantly in the training group
($P < 0.05$) as calculated by the paired t-test.
Whereas no change occurred in the control group.
Linoleic acid (18:2) and arachidonic acid (20:4)
also increased in the training subjects
($P < 0.10$) whereas in the control the mean fatty
acid had decreased the same less in the
saturated/unsaturated or saturated/total 18:2
no significant changes were obtained during training.

At present it is difficult to say whether these
exercise induced changes have any importance in
relation to health or development of atherosclerosis.
Further fatty acid analyses of postlipid
samples are needed before this problem can be
clarified.

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The Effect of 3'-5'-cyclic Adenosine Monophosphate (cAMP) on Glucose Transport in Adipose Tissue and Soleus Muscle of the Rat

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Insulin has been shown to induce decrease in the cAMP content of its target cell but little is known about the significance of this effect for the action of the hormone on glucose transport.

The present study was undertaken with the purpose of assessing the possible role of cAMP as a second messenger for the activation of the glucose transport system.

In whole epididymal fat pads cAMP (2mM) and insulin (1000U/ml) stimulated the conversion of 3-¹⁴C labelled D-glucose into CO₂ and triglycerides by 30% and 48% respectively. When added together at the same concentrations an increase of 180% was obtained. Adrenaline (10⁻⁵M) stimulated glucose metabolism by 117% in the absence and by 14% in the presence of insulin (1000U/ml).

Glucose transport was assessed by measuring the efflux of ¹⁴C 3-O-methylglucose (¹⁴C 3-O-MG) from preloaded tissue (Claussen 1969). In whole epididymal fat pads both cAMP and insulin stimulated ¹⁴C 3-O-MG efflux and the effects were additive (Table 1).

Adrenaline and salbutamol which stimulate adenylate cyclase both induced stimulation of ¹⁴C 3-O-MG efflux. Cytochalasin B abolished the effect of these stimuli indicating that it affects activation of carrier-mediated ¹⁴C 3-O-MG transport.

In soleus muscle cAMP (2mM) did not interfere

with the effect of insulin (1mU/ml) on ¹⁴C 3-O-MG efflux. Adrenaline (10⁻⁵M) and salbutamol (10⁻⁵M) stimulated ¹⁴C 3-O-MG efflux by 68% and 50% respectively.

These data argue against the idea that decrease in the cytoplasmic level of cAMP mediates the action of insulin on glucose transport in its major target tissues.

Table 1. The effects of cAMP on ¹⁴C 3-O-MG efflux from epididymal fat pads.

| | fractional loss of ¹⁴ C 3-O-MG/min (%) | |
|---|---|----------|
| Control | 0.013 ± 0.006 (8) | |
| Adrenaline (10 ⁻⁵ M) | 0.020 ± 0.001 (3) | p < 0.01 |
| Adrenaline (10 ⁻⁵ M) + insulin (250U/ml) | 0.023 ± 0.002 (3) | p < 0.01 |
| Salbutamol (10 ⁻⁵ M) | 0.020 ± 0.004 (4) | p < 0.05 |
| cAMP (0.1mM) | 0.015 ± 0.001 (4) | p < 0.2 |
| cAMP (0.5mM) | 0.020 ± 0.001 (4) | p < 0.01 |
| cAMP (1.0mM) | 0.018 ± 0.001 (5) | p < 0.05 |
| cAMP (2.0mM) | 0.017 ± 0.001 (10) | p < 0.01 |
| Insulin (250U/ml) | 0.029 ± 0.002 (3) | p < 0.01 |
| cAMP (2.0mM) + Insulin (250U/ml) | 0.021 ± 0.001 (3) | p < 0.05 |

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The Role of Ca⁺⁺ Ions in the Activation of the Glucose Transport System in Muscle and Adipose Tissue

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A wide variety of agents mimic the action of insulin in stimulating glucose transport. The purpose of the present study has been to examine the significance of calcium in the mechanism of action of these compounds.

All experiments were performed in intact soleus muscle or whole epididymal fat pads. The function of the glucose transport system was assessed by measuring the fractional loss of ¹⁴C or ³H-labelled 3-O-methylglucose (3-O-MG) from preloaded tissues. The fractional loss of ⁴⁵Ca was used as an indicator of the cytoplasmic Ca⁺⁺ level (Claussen, Elbrink & Dahl-Rasmussen 1975).

12 different agents which were found to produce from 2 to 6 fold stimulation of cytochalasin B suppressible 3-O-MG efflux all induced a highly significant (p < 0.001) increase in the fractional loss of ⁴⁵Ca both in muscle and fat pads. In each case the loss of ⁴⁵Ca preceded the stimulation of 3-O-MG efflux.

Preloading with combination of ⁴⁵Ca and ³H 3-O-MG allowed direct comparison between the changes in the fractional loss of the two isotopes from individual muscle. When tested at different concentrations the 5 agents listed in Fig. 1 induced dose-dependent increases in the fractional loss of ⁴⁵Ca- and ³H-activity. Regression

analysis showed significant positive correlation between these two parameters.

The results support the idea that in the major targets for insulin action Ca⁺⁺ ions participate in the mechanism of activation of the glucose transport system.

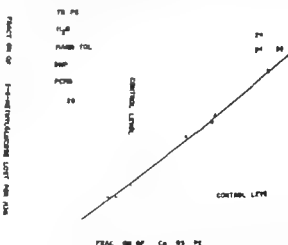


Fig. 1. Effect of glucose transport stimulants on the efflux of fractional loss of ⁴⁵Ca and ³H 3-O-MG from soleus muscles. Each point represents mean of 3 observations.

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the influence of autonomic agents and hypoxia on gas exchange is performed in fish gill

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Blood enters the complex gill vasculature through afferent arteries distributing into secondary lamellae where gas exchange occurs. Leaving the lamellae blood proceeds to the arterial systemic circulation via afferent filament and branchial veins. Alternatively post lamellar blood may enter nutritive vessels of the gill branchial system or proceed through anastomosis to the branchial venular vein.

Earlier studies have demonstrated that catecholamines favour flow in the afferent arterial system causing reduced \rightarrow flow. Such a flow shift is likely to raise the O_2 transport efficiency of the gills at times of increased ventilatory activity. The objective of this study was to assess if adrenaline and acetylcholine (ACh) affect the oxygen level of the perfusate in afferent gill preparation. Additionally the effects of ambient and perfusate O_2 tensions on the gill vascular resistance have been studied.

The gill apparatus of the cod *Gadus morhua* was perfused in situ via the ventral aorta. The dynamics of the perfusion fluid and ambient water was regulated. The gas mixing pumps arterial and venous outflow and their O_2 tensions were continuously monitored as was ventral aortic perfusion pressure.

At 10^{-5} M increased both volume and O_2 of the arterial outflow by 40% and 45% res-

pectively while concurrently the venous effluent pO_2 and outflow declined. Ventral aorta pressure fell indicating reduced gill vascular resistance following adrenaline administration.

ACh (3×10^{-4} M) caused only very small changes in the flow of both effluents but a drop in both arterial and venous pO_2 . The increased pO_2 of the arterial outflow associated with adrenaline injection is likely related to microcirculatory re-arrangement involving lamellar recruitment. The increased gill vascular resistance with ACh administration may result from pillar cell contractions elevating lamellar resistance.

Hypoxic conditions induced by lowering perfusion fluid pO_2 or ambient pO_2 resulted in elevated ventral aorta pressure. The site of the increased resistance to hypoxia is probably the same as that for ACh since this agent gave only a slight additional increase in branchial resistance compared to the response in a non-hypoxic situation.

Cerebral blood flow and ^{14}C -deoxyglucose accumulation in the brain

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In the CNS the metabolism is correlated to the activity both temporally and spatially. The blood flow is under strong influence of local factors and as consequence one can expect that under normal condition there is a good correlation between local activity and local blood flow. Good evidence for such correlation has been presented e.g. by Ingvar and Lassen (1977).

In experimental animals local blood flow can be measured with the microsphere method.

Local cerebral glucose consumption can also be expected to reflect local activity in the CNS glucose being the main nutrient. This presumption that oxidative metabolism dominates by far regional with anaerobic glycolysis might have low metabolism and still high glucose consumption.

A method for studies on local glucose consumption was devised by Sokoloff (1975, 1977) the assumption being that accumulation of deoxyglucose which enters the cells very much like glucose and is then phosphorylated but not metabolized any further reflects glucose consumption.

We have investigated the relation between regional blood flow as measured with microspheres and deoxyglucose accumulation in 9 regions in one anesthetized rabbit and 17 regions in one monkey under pentobarbital anesthesia. After the

preparation of narrow fraction of 9 μ m microspheres these were injected into the left heart ventricle about 40 in the injection of ^{14}C -deoxyglucose the animal was killed and the brain frozen and sectioned. The deoxyglucose concentration in sections was determined using densitometry of autoradiographs. The number of spheres was determined in the same regions by counting in a scintoscope.

In both animals there was four to five-fold variation in deoxyglucose concentration from posterior white matter to the grey matter of the inferior colliculus. The variation in sphere concentration was about 20-fold. The relationship between concentration of deoxyglucose and the concentration of spheres appeared nonlinear and seemed to be best fitted by an equation of the form $CD = k \cdot VCS$. Both methods thus seem to give measures reflecting local metabolism but the coupling between local blood flow as measured with μ m spheres and deoxyglucose accumulation probably varies from one part of the brain to another.

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Brain uptake of mannitol and sucrose after cerebral ischemia in normo- and hyperglycemic rats
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The permeability of the cerebral capillary endothelium is not affected by a short lasting ischemic episode from which the animals recover completely. However, pre-treatment of such animals with glucose causes them to succumb completely after ten minutes of global ischemia (Sienkiewicz & Hansen 1978). The absent restitution may be a consequence of increased blood-brain barrier permeability affecting ion and neurotransmitter homeostasis. In order to investigate this possibility the permeability of the capillary endothelium to mannitol and sucrose was studied after ten minutes of global cerebral ischemia. Normo- or hyperglycemic rats were strangled by inflation of a pneumatic cuff and reduction of arterial blood pressure to 50 mmHg. Plasma glucose was changed prior to ischemia by i.p. glucose administration.

The regional permeability-surface area product (PS) was measured after i.v. bolus injection of 14 C-mannitol and 3 H sucrose by the integral method. The PS for mannitol averaged 0.17 ± 0.02 (sd) ml/(100g) min in normoglycemic rats before ischemia and the mannitol sucrose

ratio averaged 2.3 ± 0.5 .

When plasma glucose was raised to 28 mM PS for mannitol decreased to 0.09 ± 0.02 and the mannitol:sucrose ratio remained unchanged (2.6 ± 0.1).

Immediately after ischemia PS for mannitol increased 2.3 fold above the respective control values but two hrs after ischemia PS was again reduced to 80% of control values in the normoglycemic rats and slightly above control values in the hyperglycemic rats. The mannitol:sucrose ratios were never significantly different from the respective control values.

The results indicate that the permeability of the cerebral capillary endothelium to small polar solutes is unchanged after ten minutes ischemia. It is therefore unlikely that the detrimental effect of hyperglycemia on the outcome of cerebral ischemia is a consequence of decreased integrity of blood brain barrier.

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D 14

Inotropic effect of salbutamol and prenalterol on pig myocardium in vitro

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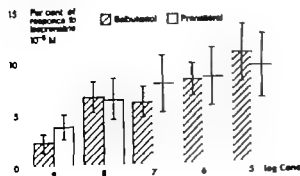
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Salbutamol is a β_2 -adrenoceptor stimulator (Cullum et al. 1969) used clinically in the treatment of asthma and prenalterol (like the racemic H 80/62) is a β_1 stimulator with marked positive inotropic action on the heart (Carlsson et al. 1977 & Johnsson et al. 1978). A series of experiments was planned to

classify the β -adrenoceptor in different tissue from pigs using these selective adrenergic agonists. Contractile force was recorded in electrically stimulated strips or trabeculae of porcine myocardium mounted in vitro in standard Krebs solution. Salbutamol in concentrations up to 10^{-5} M exerted a expected only a weak inotropic influence; the maximum increase in active force was $11 \pm 4\%$ (mean \pm SE) of the response to isoprenaline 10^{-6} M. However, prenalterol in dose up to 10^{-6} M had a weak action $10 \pm 3\%$ relative to isoprenaline. This low intrinsic efficacy of prenalterol in these experiments contrasts with its documented action in vivo and may be due to species difference or to the unphysiological conditions in vitro. The present in vitro study therefore does not conclusively classify the porcine myocardial β -adrenoceptor as β_1 or β_2 predominance.

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lymph and brain uptake of EDTA sodium and
 made in 1972.
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The blood-retinal barrier (BRB) is considered an
 analog to the blood-brain barrier (BBB). It is
 known that small water-soluble substances such
 as sodium fluorescein (Grayson & Laties 1971)
 and mannitol (Smith & Rudt 1975) and sodium
 fluorescein (Grayson & Laties 1971) do
 not easily pass the BRB, but the permeability char-
 acteristics of the BRB are less well known than
 those of the BBB. The present study was undertaken
 to compare the penetration into and through the
 BRB and the BBB by determining the concentrations
 of various substances in retina and brain after an
 intracarotid injection.

The intravascular concentrations of ^{51}Cr -EDTA
 were determined in retina and brain 60-90 sec
 after injection in albino rats. In these experiments
 ^{51}Cr -albumin was included as an intravascular marker
 and the activities due to ^{125}I -I and ^{51}Cr in
 weighed blood samples were used to calculate and
 correct the activity due to ^{51}Cr -EDTA located in
 the intravascular space. By comparing the intra-
 vascular concentrations of ^{51}Cr -EDTA ratio
 between the permeability surface area (PS) pro-
 ducts for retina and brain was obtained. This ratio
 was about 8. To determine whether this was due
 to higher permeability or larger surface area
 of the BRB the rate constants for the wash-out of
 freely diffusible substance from retina and
 brain was determined. This was done by determining

the fraction of injected ^{51}Cr -EDTA recovered in weighed
 samples of retina and brain at various times after
 an intracarotid injection as before. The rate
 constant for the BRB was about 7 times that for the
 BBB indicating that larger surface area is the
 main factor determining the difference in PS-pro-
 ducts between the BRB and the BBB.

In third series of experiments, the concentrations
 of ^{22}Na and ^{125}I in retina and brain 5-7 sec
 after intracarotid injection were compared. The reti-
 nal concentrations were 5-9 times that in the brain
 indicating that also ions enter the retina more
 easily than the brain. The concentrations of ^{22}Na
 and ^{125}I in the brain were the same while in the
 retina the ratio Na/I was 1.4. Transport mechanisms
 or electrical forces may explain this difference
 in retinal uptake of Na and I .

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Transport across the blood-retinal barrier
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In analogy to the blood-brain barrier (BBB) there
 is also blood-retinal barrier (BRB) located in
 the retinal capillary wall and the retinal pigment
 epithelium. Metabolic substrates are transported
 across the BRB by carriers exhibiting saturability
 and stereo-specificity. The aim of the present study
 was to compare the passage of hexoses through
 these two barriers.

The brain uptake index (BUI) method (Oldendorf 1971)
 was modified to obtain also the retinal uptake in-
 dex (RUI). Thus RUI and BUI were determined for
 various monosaccharides alone and in the presence
 of 40 mM unlabeled D-glucose (carrier-inhibition).
 RUI and BUI were also determined for D-glucose
 inhibited by unlabeled D-glucose in concentrations
 between 1 and 300 mM (self inhibition).
 The carrier with the same relative affinity to
 several hexoses was found for both barriers. The
 results obtained with self inhibition of D-glucose
 were used to calculate the K_m -values for the trans-
 port across the BRB and BBB. K_m -values calculated
 for self inhibition up to 3 mM were 13.7 and 3.5
 mM for BRB and BBB respectively. However, they in-
 creased gradually with increasing concentrations
 of unlabeled D-glucose above 3 mM. Two possible
 explanations are accelerated exchange diffusion
 and two transport systems. It was concluded that
 accelerated exchange diffusion of glucose across
 the BRB and the BBB is the most likely explanation.

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Effects of substance P, PGE₂ and aspirin on the pupillary sphincter modification by tetrodotoxin

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The rabbit eye is very useful in studies on inflammatory processes. Two kinds of mechanisms have been particularly studied: one involving the release of prostaglandins and the other involving a noncholinergic neural component.

Prostaglandins in the eye produce miosis as well as intraocular pressure and breakdown of the blood-aqueous barrier. Neuronal irritation produced e.g. by antidromic stimulation of the trigeminal nerve or administration of irritant such as capsaicin has similar effects. The neuronal effect seems to be due to release of substance P. Similar substance (Bill et al 1979).

We have investigated the effects of substance P, PGE₂ and aspirin on the pupil size and what extent the effects require normal nerve function. Intracranial injection of 10 µg of tetrodotoxin (TTX) on one side was used to block nerve conduction in that eye. At this dose antidromic stimulation of the trigeminal root had practically no effect on the pupil size indicating effective blockade.

The dose-response relationship of intracranial substance P was not significantly affected by TTX, slight contraction of the sphincter pupillae occurred at 200-400 pg and miosis at

1000-2000 pg.

PGE₂ doses of 15-40 µg produced moderate miosis on the control side but did not have distinct effects on the pupil size on the TTX side. Capsaicin 30-120 µg was strongly miotic on both sides.

Butt and Hammond (1977) demonstrated that the trigeminal denervation of the eye, PGE₂ and capsaicin have no miotic effect. But substance P is strongly miotic (Butt, unpublished).

It is most likely then that the miosis caused by substance P is due to direct effect on the sphincter pupillae muscle. The PGE₂ miosis requires neuronal impulse propagation and thus is not direct muscular effect. PGE₂ miosis could be due to activation of prostaglandin receptors on the peripheral nerve endings and subsequent release of substance P. Capsaicin miosis requires presence of nerve but can be produced even after blockade of nerve conduction suggesting that this substance causes release of substance P via a similar substance by an unspecific effect on the permeability of the nerve endings.

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D 18

EFFECTS OF FACIAL NERVE STIMULATION ON OCULAR BLOOD FLOW AND THE INTRAOCULAR PRESSURE (IOP) IN THE CYNOLOGUS MONKEY

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Morphological evidence of a parasympathetic nerve supply via the facial nerve to the ocular and cerebral blood vessels. We have studied the effect of stimulation of the facial nerve in rabbits on vasodilation in the eye and an increase in IOP. Blood flow also increased in the submandibular gland. The effects on the eye are similar to those of cholinergic and ganglionic blockade (Stjerneschwantz & Bill 1980) which indicates that the vasodilation is caused by non-conventional transmitters. One possible candidate is vasoactive intestinal polypeptide (VIP). Intravenous injection of VIP in rabbits caused an increase in ocular blood flow and IOP (Nilsson & Bill 1979).

In the present study region 1 blood flow was determined in cynomolgus monkeys (n=7) using the labelled microsphere method. The facial nerve on one side was exposed, sectioned and intracranially stimulated. The ipsilateral acoustic pupal IOP was registered in both eyes. Three blood flow measurements were made in each monkey: one without stimulation and two during stimulation before and after cholinergic (tropine) or ganglionic (hexamethonium) blockade.

In 4 other monkeys VIP was injected and the effects on the blood pressure and IOP were studied.

Ocular blood flow on the stimulated side increased by about 100% during stimulation. IOP also increased on the stimulated side. The effects were reversed by atropine blockade but were completely abolished after ganglionic blockade. Cerebral blood flow was not affected during stimulation. The blood flow in the submandibular gland increased 3-20 times. This effect was markedly reduced by cholinergic blockade and abolished after ganglionic blockade. Injections of VIP caused a decrease in blood pressure and an increase in IOP.

The results indicate that the facial nerve stimulation causes vasodilation in the eye via the nerve that carries the parasympathetic output between the eye stimulation and the peripheral nerve terminal and they suggest that VIP or some other unconventional transmitter is involved in the response.

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and analgesic effects of 5-hydroxytryptamine (5-HT) agonist.

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firing of ascending 5-HT neurons is depressed by stimulation of presynaptic receptors α by administration of low doses of the 5-HT agonist 5-hydroxy- α -N-dimethyltryptamine (5-MeO-DMT) (Jensen Jacobs 1979) which has preferential action on presynaptic 5-HT receptors (De Montigny & Aghajanian 1977). Higher doses of 5-MeO-DMT have postsynaptic agonist effects as well (Jensen et al 1977) depressing the response to nociceptive heat in the tail-flick (TF) test (Jensen 1979). In the present study we investigated if stimulation of presynaptic and postsynaptic receptors with 5-MeO-DMT could be demonstrated for the descending 5-HT pathways using the sensitivity to nociceptive heat as test for the function of these neurons (Jensen & Nolle 1978).

5-MeO-DMT (a volume of 25 μ l was injected into the lateral ventricle of rats μ -0 (per each dose, low doses 0.6, 1.6, 3.2, 6.4 and 12.8 μ g) produced hyperalgesia (maximal reduction of TF latency 24%). Fifty and 100 μ g caused hyperalgesia (hyperalgesia followed by analgesia). A dose of 400 μ g resulted in long-lasting (more than 30 min) analgesia (mean TF latencies 130-140% of preinjection scores).

Taken together with previously reported data relating modulation of pain sensitivity via spinal 5-HT receptors (Jensen 1979) these

results suggest that also in the descending 5-HT system, 5-MeO-DMT in low doses preferentially stimulates presynaptic receptors leading to hyperalgesia due to reduced activity in descending fibers. Higher doses however cause analgesia probably by stimulating postsynaptic 5-HT receptors in the spinal cord.

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D 24

The affinity uptake of glutamate in terminals of corticospinal pathway

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Glutamate is accumulating that glutamate (Glu) may be the transmitter in several corticospinal and corticobulbar pathways and that high affinity uptake of 3 H-Glu may be useful for tracing such pathways (e.g. Parnas et al. 1979). The putative spinal nucleus dorsalis projection of fibres from the cerebral cortex. This excitatory projection (Sasaki et al. 1970) is predominantly unilateral and appears to constitute by far the major input of these nuclei (see Brodal 1972).

To corticospinal fibres were covered in rats by transecting the cerebral peduncle just rostral of the suboccipital sign by means of tongs. The rats were sacrificed about 2-3 w after at right angle through the tip of cerebellum that was lowered stereotactically across the ventrolateral surface of the peduncle. The animals were killed and the putative spinal nucleus dorsalis projection of fibres from the cerebral cortex. This excitatory projection (Sasaki et al. 1970) is predominantly unilateral and appears to constitute by far the major input of these nuclei (see Brodal 1972).

At 5 days after bilateral lesions the Glu uptake was reduced to 25-30% (mean \pm SEM, 8 samples from 4 animals, $P < 0.001$) of the uptake in sham-operated rats. 7 samples from sham operated controls survived for 7 or 12 days resulted in no further

reduction. The value for GABA was $95 \pm 9\%$. Similar results were obtained when 3 H-D-aspartate was substituted for 3 H-Glu. At 5 days after unilateral transection Glu uptake was $42 \pm 6\%$ ipsilateral and $77 \pm 2\%$ contralateral to the lesion.

These results show that afferents to the putative spinal nucleus dorsalis projection of fibres from the cerebral cortex possess Glu uptake capacity in their terminals. The fibres may be partly crossed. Terminals that take up GABA are predominantly of other origin.

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Effect of adrenalin on the tentacle reflex of *Helix pomatia*

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It is a common feature of contraction of many vital invertebrate organs that the speed and degree of contraction changes with repetitions of the initiating stimulus (Horn & Hinde 1970; Kandel 1979). These movement changes possess a large number of properties that are qualitatively identical to learning phenomena in higher animals. Further they are controlled by simple even monosynaptic neural networks in which the plastic element has been localized to the synapse and in one case (Kandel 1979) described a presynaptically caused depression (Christoffersen & Juel 1979). As a supplement to the observation of a series of learning-like properties of the tentacle withdrawal of *Helix pomatia* (Christoffersen in prep.) one more feature of learning known from higher animals was presently tested and found: sensitivity to adrenalin.

Repeated electrical stimulation of an optic tentacle led to a fall of the frequency of associated withdrawal extension sequences. A control series of stimulations of the left tentacle for one hour was followed by injection of adrenalin (body concn approx 10^{-6} M). Thereafter a one hour test series was performed on the

right tentacle. The reflex plasticity in the two tentacles is independent wherefore control and test series can be performed in the same animal.

In 5 test experiments a significant elevation of the frequency curve was observed before injection of the area under the mean curve was 135 ± 8 (S.D.) stimulations as opposed to 185 ± 26 (N.D.) after injection. The students-t test of the increase of the area gave $P = 0.03$. The injection of pure Ringer in 4 control animals did not affect the curve significantly. The elevation may be described as an increase of activity most likely due to enhancement of cardiac output (Dale 1973). Such activity change is generally known to take place and affect learning in vertebrates (Gold & McGaugh 1975).

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D 22

On the control of the central pattern generators for swimming in the isolated lamprey spinal cord *in vitro*

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The thin (200-300 μ m) spinal cord of the lamprey (*Ichthyomyzon unicuspis*) can be maintained *in vitro* for several days in cooled Ringer. If descending fibres in the cord are electrically stimulated or if glutamic acid (0.6-1.2 μ M) is applied to the bath coordinated motor activity can be recorded in the ventral roots (Cohen & Wallén 1978; Poon 1979). Bursts of motoneuronal discharge alternate between the two sides of each segment. The activity along the cord is coordinated with a constant phase lag between adjacent segments. These are the characteristics of swimming in fish and accordingly the spinal network can be said to generate fictive swimming (without movement). The motoneurons undergo periods of depolarisation and hyperpolarisation (IPSPs) (Russell & Wallén 1979). The burst rate is markedly influenced by the pool temperature. It could be changed between 3°C and 25°C (Typically kept at 7°C).

The central network appears to depend on post synaptic inhibitory mechanisms. Application of the glycine antagonist strychnine influences the rhythmic activity. When the dose is increased in several steps from 0.5 to 2.5 μ M the frequency of alternation increases progressively. At the highest dose the rhythm becomes very variable and bursts may be skipped. Finally tonic activity occurs on both sides without reciprocal interactions. The putative

inhibitory transmitter glycine (1 μ M in the bath) not only decreases the degree of afferent activity but also lowers the rate of bursting and will finally abolish the rhythm. GABA another putative inhibitory transmitter is less likely to be involved as its antagonist bicuculline (0.025-2 μ M/L) does not seem to influence the rhythmic activity to any significant degree.

Mechano-receptors in or around the notochord can profoundly influence the central network controlling swimming. One part of the notochord with the spinal cord was bent back forth while the other part was fixed in the dish. This caused the rhythm to be modified and follow the imposed movement. A movement towards the left side would induce left side activity and vice versa. The receptors must be located in the notochord with adjacent connective tissue as muscle and skin have been removed. Possible receptors in the spinal cord itself can be excluded as a transection of dorsal and ventral roots abolishes the driving effect. These feedback effects are analogous to those of the dogfish (Grillner & Wallén 1977) and are similar to those of the cat hindlimb (Andersson et al 1978).

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Hyper- and hypoalgesic effects of 5-Hydroxytryptamine (5-HT) agonists.

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First, if ascending 5-HT neurons are depressed by stimulation of presynaptic receptors a hypoaesthesia of low doses of the 5-HT agonist 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) (Tran Jacobs 1979) which has preferential action on presynaptic 5-HT receptors (De Montigny & Aghajanian 1977). Higher doses of 5-MeO-DMT have postsynaptic agonistic effects as well (Tran et al. 1977) depressing the response to nociceptive heat in the tail flick (TF) test (George 1979). In the present study we investigated if stimulation of presynaptic and postsynaptic receptors with 5-MeO-DMT could be demonstrated for the descending 5-HT pathways using the sensitivity to nociceptive heat as test for the function of these neurons (George 1979).

5-MeO-DMT in volume of 25 µl was injected into the lateral ventricle of rats (n=6 for each dose, low doses (1.6, 3.2, 6.4, 12.8 and 25.6 µg) produced hyperalgesia (baselines reduction of TF latency 24). Fifty and 100 µg caused biphasic response: hyperalgesia followed by analgesia whereas 400 µg resulted in long-lasting (more than 90 min) analgesia (mean TF latencies 130-140% of preinjection scores).

Taken together with previously reported data indicating modulation of pain sensitivity via spinal 5-HT receptors (George 1979) these

results suggest that also in the descending 5-HT system, 5-MeO-DMT in low doses preferentially stimulates presynaptic receptors leading to hyperalgesia due to reduced activity in descending fibers. Higher doses however cause analgesia probably by stimulating postsynaptic 5-HT receptors in the spinal cord.

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D 24

High affinity uptake of glutamate in terminals of the corticospinal pathway

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Glutamate is accumulating that glutamate (Glu) may be the transmitter in several corticocortical and corticospinal pathways and that high affinity uptake of [³H]-L-Glu may be useful for tracing such pathways (e.g. Fournier et al. 1979). The postcortical nuclei receive dense projection of fibres from the cerebral cortex. This excitatory projection (Brodal et al. 1970) is predominantly unilateral and appears to constitute by far the major input of these nuclei (see Brodal 1977).

The corticospinal fibres were severed in rats by transecting the cerebral peduncle just rostral of the substantia nigra by means of a tungsten wire extended about 2.5 mm at right angle through the tip of cerebellum that was lowered stereotactically across the ventrolateral surface of the peduncle. The animals were killed and the postcortical nuclei were dissected under the microscope from fresh slices and homogenized in buffered 0.3 M sucrose to produce suspensions of nerve ending particles. High affinity uptake of [³H]-L-Glu and [³H]-GABA (2 µM each) was measured by double labelling and protein content determined as described before (Stormshagen & Stormshagen 1979).

At 5 days after bilateral lesions the Glu uptake was reduced to 35-50% of control, & samples from 4 animals (P < 0.001) of the uptake in simultaneously sampled samples from sham operated controls. Survival for 7 or 12 days resulted in no further

reduction. The values for GABA was 95 ± 9%. Similar results were obtained when [³H]-D-aspartate was substituted for [³H]-L-Glu. At 5 days after unilateral transections Glu uptake was 43 ± 6% ipsilateral and 77 ± 24 contralateral to the lesion.

These results show that afferents to the postcortical nuclei descending through the cerebral peduncles possess Glu uptake capacity in their terminals. The fibres may be partly crossed. Terminals that take up GABA are predominantly of other origin.

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Post tetanic stiffness in isolated frog muscle fibres
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Recent X-ray diffraction data (Yagi et al 1977) indicate that the return of the cross-bridges from the vicinity of the thin filaments after a contraction occurs in two stages: A rapid return of the majority of the cross-bridges was followed by a slow return of the remaining 20%. At a temperature of 4°C the slow return stage started about 1 s after the end of the stimulation and even after 5 s 10% of the cross-bridges had not yet returned.

The purpose of the present experiments was to examine whether the return to the low resting value of the stiffness of the fibre followed a similar two-stage behaviour. In this way one would obtain an indication whether the slow return of the cross-bridges reflected a detachment to the thin filaments. The experiments were carried out on 8 isolated semitendinosus muscle fibres (R. temporaria) under conditions similar to those of Yagi et al: sarcomere length 2.2 μ m, temperature 4°C, tetanic stimulation for 1 s at a frequency of 20 Hz. The stiffness was measured by subjecting the fibre to a quick stretch (ramp-and-hold) during the stimulation period and at various times (up to 5 s) after the last stimulus. In addition the stiffness of the resting fibre was measured as a reference.

At the time of the last stimulus (t = 0) the stiffness of the fibre was about 200 times the resting value. The stiffness followed the decrease of the contractile force in an almost proportional manner. At t = 1 both force and stiffness were only a few (3-5) per cent of the value measured at the time t = 0. From about t = 2.5 the contractile force was less than 0.5% of the stimulus-measured

value at t = 0 and the stiffness was only about 1.2 times the resting value corresponding to about half per cent of the value obtained at t = 0. There was no noticeable change in the stiffness during the interval 1.5-5 s. After two minutes the stiffness had returned to its resting value.

The attachment of cross-bridges such as indicated by the stiffness of the muscle fibre in the post-tetanic period thus did not follow the time course of the number of cross-bridges in the vicinity of the thin filaments. It therefore appears as if the cross-bridges can be in the vicinity of the thin filaments without being attached to these. X-ray data for the fibre being examined only give an upper limit for how great a number of the cross-bridges are attached.

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II 26

Training and the paradoxical influence of verbal encouragement on muscle fatigue

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18 healthy young male subjects performed 150 repeated maximum voluntary isometric contractions (MVC) with one leg alone (right and left leg) and with both legs simultaneously before and after a training period of five weeks duration. 5 subjects performed the functions of controls (no control group). 7 subjects performed 150 two-leg MVCs (two-leg group) and 6 subjects performed 150 one-leg MVCs (one-leg group) three times a week. In all series one contraction was performed every fifth second. Before and after the training period force and rectified smoothed electromyography (rEMG) were registered during verbal encouragement before and during each contraction. During the training period force was registered while the subjects were working alone. Results concerning training effects on strength have been given (Rube et al 1979). Results on muscle fatigue are presented here.

Before the training period force and rEMG decreased in all subjects by 2% and 33% respectively during the 150 repeated MVCs. The ratio between force and rEMG remained almost constant.

At the beginning of the training period

the initial force values were not statistically different from those of the initial values obtained during the encouragement contractions. Force decreased by 5% and 10% for the two and the one leg groups respectively.

After the first 100 repetitions the control group on an average showed a 20% decrease in force during the 150 MVCs. The two-leg group showed an unchanged decrease in force during repeated one-leg extensions but fatigue developed during repeated two-leg extensions was reduced by about 50%. In the one-leg group fatigue seen during repeated two-leg extensions remained practically constant while one-leg strength reduction was 36% smaller than prior to the training period.

The results suggest that the fatigue developed during repeated supervised and verbally encouraged MVCs is paradoxically influenced by the presence of the instructor. Furthermore that training reduces this type of fatigue is the specific type of contractions practised.

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renal reabsorption of Na and K in Gallus
role of urinary precipitate

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Determinations of urate Na and K in whole
urines and their supernatants in birds
have produced [Na]/[urate] and [K]/[urate]
molar ratios significantly greater than
mammals (McLabb, McLabb and Rinton 1973;
Aron 1974). In conjunction with measure-
ments of glomerular filtration rate and
plasma electrolyte concentrations these
findings indicate that renal reabsorption
of filtered loads may be as low as
e.g. 0.1% for Na in turkeys in con-
trast to values above 0.9% for the single-
phase urines of mammals.

In the present study single determined
ratios were made directly on dissolved
urinary precipitates of 6 hens. Urine
collected by catheters seven days after
operation and stored at 39.5°C (body tem-
perature) was centrifuged at 10
times 100 000 gravity. Precipitates
dried for 2 days over CaCl₂ were analysed
for [urate] by uricase digestion and for
[Na] and [K] by flame spectrophotometry
with standards from 5 to 8.5 mEq/L.
[Urate] in these solutions ranged from
0.2 to 5.4 mEq/L. [Na] and [K] from 0.1 to
0.6 mEq/L. Average [Na]/[urate] and [K]/
[urate] molar ratios for 31 samples are
0.112 ± 0.016 and 0.100 ± 0.013 (S.E.M.).

respectively; correction for Na and K in
precipitate due to estimated trapped
volume of supernatant produces still
lower values. The low molar ratios are
in accord with x-ray crystallographic data
reporting that avian urinary precipitates
are primarily uric acid dihydrate with
little or no contribution of urate
(Lonsdale and Suto 1971).

The molar ratios are independent of
urocrit (UT) the ratio of dry precipi-
tate to whole urine weights. UT ranges
from 0.8% in diuretic birds fed commercial
diet to 1.8% in non-diuretic birds fed a
high protein diet. Likewise molar ratios
showed no correlation with urinary [Na]
(range 2.164 mEq/L) [K] (6.78 mEq/L)
[urate] (3-265 mEq/L) in whole urine and
supernatants or with urinary pH (4.5-6.9).

Our results indicate no significant
trapping binding of Na or K in precipi-
tated urates in hen's urines. Renal
reabsorption of Na and K in the 20 samples
examined in these hens is 0.982 ± 0.003
and 0.794 ± 0.014 respectively and imply
a minor role for post-renal reabsorption of
these ions during local storage of urine.

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D 28

Metabolic adaptation in man following
prolonged exposure to high altitude

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In the acclimatization to chronic hypoxia many
adaptations occur in organs and tissues. Some of
these also occur in the skeletal muscle. It has
been suggested that denser capillary network and
an elevated oxidative potential of the muscle
secondary prolonged exposure to hypoxia (Cassia
et al. 1971; Tappin and Rasmussen 1977; Valdivia
1978). This concept has however recently been
at least partly challenged (Stiller and Sanchez-
de-
1977).

A unique possibility to study the long term adap-
tation of lowlanders to high altitude occurred in
connection with the building of an irrigation sys-
tem in the Andes. Nine men were studied before
(sea level) and after 6-44 weeks at altitude
(3500 m, bar pressure ≈ 510 mmHg). Muscle
biopsies from the leg muscles were analysed for
fibre composition and size, capillarization and
enzyme level. The physical working capacity of
each subject was also determined.

Fibre composition did not change at altitude.
The mean number of capillaries per fibre as well as
the number of capillaries around each fibre type
was unchanged. The sizes of the muscle fibre types
were smaller in 6 subjects. Consequently with
unchanged capillary to fibre ratio capillary den-
sity tended to increase and the area supplied by
each capillary was reduced.

Glycolytic enzymes did not change at all, while
citrate synthetase was reduced 9% (p < 0.05) and
3-hydroxyacyl-CoA-dehydrogenase by 54% (p < 0.05). The
muscle oxygen uptake of the subjects averaged 3.12
l/min at sea level and was only 17% lower at alti-
tude.

The present results are in contrast with the tra-
ditional concept but are in line with more recent
studies. These shorter diffusion distances in the
skeletal muscle can be part of the adaptive respon-
se of lowlanders to chronic hypoxia. This is not
brought about by proliferation of the capillaries
but rather by reduction in the size of the muscle
fibres. Further hypoxia per se does not appear to
be stimulus for enhancement of the mitochondrial
enzyme level which therefore may not be part of
the process of acclimatization to high altitude.
The high values reported earlier for skeletal
muscle oxidative enzymes in highlanders might be
explained by the use of subjects with high level
of physical activity.

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Post tetanic stiffness in isolated frog muscle fibres

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Recent X ray diffraction data (Yagi et al. 1977) indicate that the return of the cross-bridges from the vicinity of the thin filaments after a contraction occurs in two stages: A rapid return of the majority of the cross-bridges was followed by a slow return of the remaining 20%. At a temperature of 4°C the slow return began after 1.5 s after the end of the stimulation and even after 5 s 10% of the cross-bridges had not yet returned.

The purpose of the present experiments was to examine whether the return to the low resting value of the stiffness of the fibre followed a similar two-stage behaviour. In this way one would obtain an indication whether the slow return of the cross-bridges reflected attachment to the thin filament. The experiment was carried out on 8 isolated semitendinose muscle fibres (R. temporaria) under conditions similar to those of Yagi et al.: sarcomere length 2.2 μ m, temperature 4°C, tetanic stimulation for 1 s at frequency of 20 Hz. The stiffness was measured by subjecting the fibre to a quick stretch (ramp-and-hold) during the stimulation period and at various times (up to 5 s) after the last stimulus. In addition the stiffness of the resting fibre was measured as a reference.

At the time of the last stimulus (t = 0) the stiffness of the fibre was about 200 times the resting value. The stiffness followed the decrease of the contractile force in an almost proportional manner. At t = 1 s both force and stiffness were only a few (3–5) per cent of the values measured at the time t = 0. From about t = 2.5 s the contractile force was less than 0.5% of the value measured

at t = 0 and the stiffness was only about 1.2 times the resting value corresponding to about half a per cent of the value obtained at t = 0. There was no noticeable change in the stiffness during the interval 2.5–5 s. After two minutes the stiffness had returned to its resting value.

The attachment of cross-bridges such as indicated by the stiffness of the muscle fibre in the post tetanic period thus did not follow the time course of the number of cross-bridges in the vicinity of the thin filaments. It therefore appears as if the cross-bridge can be in the vicinity of the thin filaments without being attached to these. X ray data for the time being can thus only give an upper limit for how great a number of the cross-bridges are attached.

Supported by grant 511 15063 from the Danish Natural Science Research Council.

Reference

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D 26

Training and the paradoxical influence of verbal encouragement on muscle fatigue

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18 healthy young male subjects performed 150 repeated maximum voluntary muscle contractions (MVC) with one leg alone (right and left leg) and with both legs simultaneously before and after a training period of five weeks duration. 5 subjects performed the function of controls (control group). 7 subjects performed 150 two leg MVCs (two leg group) and 6 subjects performed 150 one leg MVCs (one leg group) three times a week. In all series one contraction was performed every fifth second. Before and after the training period force and rectified smoothed electromyography (rsEMG) were registered during verbal encouragement before and during each contraction. During the training period force was registered while the subjects were working alone. Results concerning training effects on strength have been given (Rube et al. 1979). Results on muscle fatigue are presented here.

Before the training period force and rsEMG decreased in all subjects by 25% and 33% respectively during the 150 repeated MVCs. The ratio between force and rsEMG remained almost constant.

At the beginning of the training period

the initial force values were not statistically different from those of the initial values obtained during the encouraged contractions. Force decreased by 5% and 10% for the two and the one leg groups respectively.

After the training period the control group average showed a 20% decrease in force during the 150 MVCs. The two-leg group showed an unchanged decrease in force during repeated one leg extensions but fatigue developed during repeated one leg extensions was reduced by about 50%. In the one leg group fatigue ended during repeated one leg extensions remained practically the same. One leg strength reduction was 36% smaller than prior to the training period.

The results suggest that the fatigue developed during repeated supramaximal and verbally encouraged MVCs is paradoxically influenced by the presence of verbal encouragement. Furthermore, that training induces this type of fatigue is the specific type of motor performance.

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Angiolar characteristics in human renal adenocarcinoma

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In vivo and in vitro studies with microsphere technique on induced RENAL cell carcinoma neoplasia have demonstrated that the tumors have relatively high resting blood flow, high resistance during maximal vascular relaxation and stress responses to noradrenaline (Weiss et al 1978). b) Pharmacologic studies (i.e. infusion of vasoactive drugs during angiography) on the contrary seem to support the hypothesis that tumor vessels of renal carcinoma are not able to constrict (Sulthorn et al 1976).

Against this background, surgically removed human kidneys with renal adenocarcinoma were subjected to hemodynamic investigation in an in vitro perfusion system where regional perfusion was studied by microsphere (ϕ 15 μ) tracer technique. Pressure-flow curves and dose-response curves for noradrenaline were obtained for the whole organ and regional vascular responses were obtained to maximal vascular relaxation and at two different doses of noradrenaline (1 and 3 μ g) at the end of the experiment the preparation was perfused with formalin including suspension of barium sulphate to allow for specimen and section angiography illustrating vascular architecture and volume or visualization of perfusion inhomogeneities. 2 mm thick sections of the whole organ including tumor were processed for autoradiography.

The majority of the tumor tissue was poorly perfused. From well-type scintillation analysis of well perfused tumor areas the peripheral resistance during maximal vascular relaxation was found to be three times that of renal cortex. Gradually increasing infusion of noradrenaline caused rapid and marked increase of peripheral resistance in tumor tissue which even seemed hyperreactive when compared to renal cortex. Angiography of the autoradiographed 2 mm sections illustrated discrepancy between vascular volume and perfusion as compared to renal cortex. Tumor areas were heterogeneously supplied with a regular network of fine-caliber vessels. Histopathological examinations were also undertaken trying to correlate morphology, vascular architecture and perfusion.

In summary the cross-sectional area of the precapillary resistance vessels of renal carcinoma is smaller than that of renal cortex. Further the tumor resistance vessels react strongly to noradrenaline and are probably hyperreactive compared to renal cortical resistance vessels. The indication of tumor vascular hyporeactivity in pharmacologic angiography can not be substantiated by this study but the discrepancy is probably due to the vastly different levels in the vascular arterioles being studied.

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ION CONDUCTANCE CHANGES IN HIPPOCAMPAL NEURONES

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When a short current pulse is injected in a neurone the membrane potential is changed and this change may be used to calculate the membrane resistance. However the voltage response to hyperpolarising current often deviates from the passive response expected from the membrane. This non-linear response was first described by Ito and others in cat alpha motoneurons. They suggested that the non-linearities were produced by mechanisms different from the mechanism causing delayed rectification and that more than one ion conductance might be involved.

We have tested if the non-linearities may be due to changes in potassium conductance only by comparing recordings from in vitro guinea pig hippocampal slices and the outcome of the simulation of a simple computer model of the nerve membrane. In the model the potassium conductance (G_K) was time and voltage dependent

$$\frac{dG_K}{dt} = G_K \{ 1 - \exp(-\frac{V - V_{rest}}{\tau}) \}$$

$$G_K \exp(k_2(V - V_{rest}))$$

$$+ G_K \exp(k_3(V - V_{rest}))$$

where V is the resting membrane potential, V_{rest} the

membrane potential and k_1 , k_2 , k_3 and τ chosen constants. Thus the potassium conductance increases during depolarisation and decreases during hyperpolarisation similar to the delayed rectification of the Hodgkin-Huxley model.

The membrane potential was calculated during and after injection of current pulses by solving

$$\frac{dV}{dt} = \frac{1}{C_m} \{ I - G_K(V - E_K) - G_{Na}(V - E_{Na}) - G_{Cl}(V - E_{Cl}) \}$$

by numerical recursive method on PDP 11/34 computer. The G are ion conductances and the E are equilibrium potentials. C_m is the membrane capacitance.

The membrane potential calculated in this model after current injections had time courses very close to the time courses recorded from CA1 in the hippocampal slice. The constants k_1 , k_2 , k_3 could be varied over a wide range without changing the basic shape of the calculated potentials. It is thus possible that the non-linearities are due to time and voltage dependent changes in only potassium conductance. Surprisingly the model also showed anomalous rectification in certain voltage ranges.

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Uterine secretory response during the reproductive cycle of the guinea pig

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In previous report(1) evidence of cholinergic presumably sympathetic secretory innervation of the guinea pig endometrium was presented. These experiments were performed on uterine brought into artificial secretory phase by combined treatment with trogen and progestrone. Uterine secretion has been calculated from amount of carbohydrate released from isolated perfused guinea pig uterine(1). Change in secretion in response to field stimulation and/or administration of drugs were calculated per cent increase above resting secretion level(1).

Since the endometrium undergoes changes according to the hormonal state of the animal and a adrenergic sympathetic nerve is subserved changes due to the serum levels of female sex hormones today possible secretory response during the normal estrous cycle pregnancy well after treatment with one of trogen and progestrone was performed.

In estrous cycle both carbachol and field stimulation increased secretion except during late diestrus. In pregnancy secretory response was noted at implantation time. From mid pregnancy and onwards response of increasing magnitude were obtained. The secretory response persisted post partum.

Furthermore evidence of an adrenergic inhibitory influence on the neurogenic cholinergic response was obtained at estrus.

Both carbachol and field stimulation increased carbohydrate secretion by about 50% above resting secretion level in uterine of animal treated with both trogen and progestrone. In uterine of animal receiving only trogen the increase was only about 20%. Secretory response were obtained in uterine of animal treated with progestrone only.

From this it could be suggested that both hormones are necessary for full cholinergic secretory response to field stimulation or to carbachol. Furthermore it could be concluded that muscarinic stimulation of the endometrium results in mucus secretion during the crucial event of the reproductive cycle at ovulation and implantation time during period of fetal growth and delivery as well as post partum (when the para ovulate and normally become fertilized again). Furthermore cholinergic secretory innervation seems to be present at all these times.

Reference

HAMMARSTRÖM M & BJÖRSTRAND M O 1979 Acta Physiol Scand 106 11-13 (1)

D 30

A comparison between freon and acetylene rebreathing for measuring cardiac output during rest and submaximal exercise

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The non-invasive rebreathing technique for measuring cardiac output (CO) has attracted renewed interest with the construction of mass spectrometers. Kruhnauer (pers comm) uses freon II replacing acetylene (Grollmann 1929) as the soluble gas. Triebwasser et al (1977) used the non soluble gas He in order to correct the fractions of the soluble gas for incomplete mixing and from a semi logarithmic plot of the corrected values CO was calculated.

We measured CO in 10 subjects during rest and submaximal exercise using rebreathing of 2.2-5.1 of 1% acetylene 3-4% freon 6 10% argon and 39-42% oxygen in nitrogen CO calculated from the two gases correlated significantly ($r=0.9923$) but freon values were systematically lower. This might be due to differences in the in vitro and the in vivo Bunsen solubility coefficients. The results were comparable with those reported in literature. Hyperventilation increased CO significantly at rest and low work rates. This is attributed to a suction on the central veins demonstrated by an increase in central venous pressure. At higher work loads there was a decrease in the rebreathing bag volume as indicated by an increase of the argon fraction due to CO₂ accumulation in the lung bag sys-

tem which causes a decreased CO₂ excretion rate while O₂ is still taken up at the same rate. This stresses the importance of using an inert gas as an indicator of incomplete mixing and volume changes because these two errors can be corrected for. The increased heart rate during rebreathing was shown to be caused by the increased PCO₂.

Conclusion: Freon can replace acetylene as the soluble gas. Spontaneous breathing is recommended. An inert insoluble gas (He or Ar) must be used in order to give reliable results.

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GROLLMANN A 1929 Am J Physiol 88 432-445
TRIEBWASSER et al 1977 Aviat Space Environ Med 48(3) 203-209

Tumor characteristics in human renal adenocarcinoma

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In vivo and in vitro studies with microsphere tracer technique as induced GHA in mammary neoplasia have demonstrated that the tumors have relatively high resting blood flow, high resistance during maximal vascular relaxation and strong response to noradrenaline (Weiss et al 1979). b) Pharmacological studies (1) infusion of vasoconstrictive drugs during angiography on the contrary seem to support the hypothesis that tumor vessels of renal carcinoma are not able to constrict (Mikhael et al 1976).

Against this background, surgically removed human kidneys with renal adenocarcinoma were subjected to hemodynamic investigation in an in vitro perfusion system where regional perfusion was studied by microsphere (15 µm) tracer technique. Pressure-flow curves and dose-response curves for noradrenaline were obtained for the whole organ and regional vascular response was obtained. Maximal vascular lumen and at two different stages of noradrenaline 1. At 3 points. At the end of the experiment the preparation was perfused with formalin including compensation of barium chloride to allow for specimen and section angiography illustrating vascular architecture and volume. For visualization of perfusion isohomocysteine in thick sections of the whole organ including tumor were processed for autoradiography.

The majority of the tumor tissues was poorly perfused. From wall type scintillation analysis of well perfused tumor areas the peripheral resistance during maximal vascular relaxation was found to be three times that of renal cortex. Gradually increasing infusion of noradrenaline caused rapid and marked increase of peripheral resistance in tumor tissues which even seemed hyperreactive when compared to renal cortex.

Angiography of the autoradiographed 2 mm sections illustrated discrepancy between vascular volume and perfusion as compared to renal cortex. Tumor areas were heterogeneously supplied with an irregular network of fine-caliber vessels. Histopathological examinations were also undertaken trying to correlate morphology, vascular architecture and perfusion.

In summary the cross-sectional area of the pre-capillary resistance vessels of renal carcinoma is smaller than that of renal cortex. Further the tumor resistance vessels react strongly to noradrenaline and are probably hyperreactive compared to renal cortical resistance vessels. The indication of tumor vascular hyperreactivity in pharmacological studies can not be substantiated by this study but the discrepancy is probably due to the vastly differing levels in the vascular arterioles being studied.

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ION CONDUCTANCE CHANGES IN HIPPOCAMPAL NEURONES

Abstract

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When short current pulse is injected in neuron the membrane potential is changed and this change may be used to calculate the membrane resistance. However the voltage response to hyperpolarising current often deviates from the passive response expected from the membrane. This non-linear response was first described by Ilk in the 1940s and later further investigated by Ilk and others in cat and non-mammalian. They suggested that the non-linearities were produced by mechanisms different from the mechanisms causing delayed rectification and that more than one ion conductance might be involved.

We have tested if the non-linearities may be due to changes in potassium conductance only by comparing recordings from in vitro guinea pig hippocampal slices and the outcome of the simulation of a simple computer model of the nerve membrane. In the model the potassium conductance (G_K) was time and voltage dependent:

$$\frac{dG_K}{dt} = G_K \cdot A(G_{Kmax} - G_K) \\ G_K \exp \{k_2(V - V_1)\}$$

$$+ G_K \exp \{k_1(V - V_2)\}$$

where V is the resting membrane potential, V_1 the

membrane potential and k_1 , k_2 , V_1 and V_2 chosen constants. Thus the potassium conductance increases during depolarisation and decreases during hyperpolarisation, similar to the delayed rectification of the Hodgkin-Huxley model.

The membrane potential was calculated during and after injection of current pulse by solving

$$\frac{dV}{dt} = \{(-G_K(V - E_K) - G_{Na}(V - E_{Na}) - G_L(V - E_L)) / C\}$$

by numerical recursive method on PDP 11/34 computer. The E are ion conductances and the V are equilibrium potentials. C the membrane capacitance.

The membrane potential calculated in this model after current injections had time courses very close to the time courses recorded from CA1 in the hippocampal slice. The constants k_1 , k_2 could be varied over wide ranges without changing the basic shape of the calculated potentials. It is thus possible that the non-linearities are due to

time and voltage dependent change in only potassium conductance. Surprisingly the model also showed anomalous rectification in certain voltage range.

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Uterine secretory response during the reproductive cycle of the guinea pig

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In a previous report (1) evidence of a cholinergic presumably sympathetic secretory innervation of the guinea pig endometrium was presented. These experiments were performed on uteri brought into artificial secretory phase by a combined treatment with oestrogen and progesterone. Uterine secretion has been calculated from amount of carbohydrate released from isolated everted guinea pig uteri (1). Change in secretion in response to field stimulation and/or administration of drugs were calculated as per cent increase above resting secretion level (1).

Since the endometrium undergoes change according to the hormonal state of the animal and adrenergic myometrial nerve are subjected to changes due to the serum levels of female sex hormones, study of possible secretory responses during the normal estrous cycle pregnancy well after treatment with one of oestrogen and progesterone was performed.

In estrous cycle both carbachol and field stimulation increased secretion except during luteal phase. In pregnancy secretory response was noted at implantation time. From mid pregnancy and onwards response of increasing magnitude were obtained. The secretory response persisted post partum.

Furthermore evidence of an adrenergic inhibitory influence on the neurogenic cholinergic response was obtained (2) and true.

Both carbachol and field stimulation increased carbohydrate secretion by about 50% above resting secretion level in uteri of animals treated with both oestrogen and progesterone. In uteri of animals receiving only oestrogen the increase was only about 20%. No secretory response were obtained in uteri of animals treated with progesterone only.

From this it could be suggested that both hormones are necessary for full cholinergic secretory response to field stimulation or to carbachol. Furthermore it could be concluded that muscarinic stimulation of the endometrium results in mucous secretion during luteal phase of the reproductive cycle, at ovulation and implantation time during period of fetal growth and delivery well post partum (when the para-ovules and normally become fertilized again). Furthermore cholinergic secretomotor innervation seems to be present at all these times.

Reference

HANMARSTRÖM M & SJÖSTRAND N O 1979 Acta Physiol Scand 106 11-15 (1)

D 30

A comparison between freon and acetylene rebreathing for measuring cardiac output during rest and submaximal exercise

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The non-invasive rebreathing technique for measuring cardiac output (CO) has attracted renewed interest with the construction of mass spectrometers. Kruhoffer (pers comm) uses freon-22 replacing acetylene (Grollmann 1929) as the soluble gas. Triebwasser et al (1977) used the non-soluble gas He in order to correct the fractions of the soluble gas for incomplete mixing and from a semi-logarithmic plot of the corrected values CO was calculated.

We measured CO in 10 subjects during rest and submaximal exercise using rebreathing of 2.5% of 1% acetylene 3-4% freon 6-10% argon and 39-42% oxygen in nitrogen. CO calculated from the two gases correlated significantly ($r=0.9923$) but freon values were systematically lower. This might be due to differences in the in vitro and the in vivo diffusion solubility coefficients. The results were comparable with those reported in literature by percent ventilation increased CO significantly at rest and low work rates. This is attributed to a suction on the central veins demonstrated by an increase in central venous pressure. At higher work loads there was a decrease in the rebreathing bag volume as indicated by an increase of the argon fraction due to CO₂ accumulation in the lung bag system which causes a decreased CO₂ excretion rate while O₂ is still taken up at the same rate. This stresses the importance of using an inert gas as an indicator of incomplete mixing and volume changes because these two errors can be corrected for. The increased heart rate during rebreathing was shown to be caused by the increased PCO₂.

Conclusion: Freon can replace acetylene as the soluble gas. Spontaneous breathing is recommended. An inert insoluble gas (He or Ar) must be used in order to give reliable results.

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vascular characteristics in human renal adenocarcinoma

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In vivo and in vitro studies with microsphere transfer technique on induced DNA but secondary neoplasia have demonstrated that the tumors have relatively high resting blood flow high resistance during arterial vascular relaxation and strong response to noradrenaline (Weiss et al 1973). b) Pharmacological studies (i. infusion of vasoactive drugs during angiography) on the contrary seem to support the hypothesis that the tumor vessels of renal carcinoma are not able to constrict (Shaloud et al 1976).

Against this background surgically removed human kidneys with renal adenocarcinoma were subjected to hemodynamic investigation in an in vitro perfusion system where regional perfusion was studied by microsphere (8-15 μ m) transfer technique. Pressure/flow curves and dose/response curves for noradrenaline were obtained for the whole organ and regional vascular responses were obtained at arterial vascular relaxation and two different doses of noradrenaline. i) points. At the end of the experiment the preparation was perfused with formalin including suspension of barium sulphate to allow for specimen and section angiography illustrating vascular architecture and volume. For visualization of perfusion intravascularly 2 mm thick sections of the whole organ including tumor were processed or autoradiography

The majority of the tumor tissue was poorly perfused. From well-type scintillation analysis of well perfused tumor areas the peripheral resistance area during arterial vascular relaxation was found to be three times that of renal cortex. Gradually increasing infusion of noradrenaline caused rapid and marked increase of peripheral resistance in tumor tissue which even seemed hyperreactive when compared to renal cortex.

Angiography of the autoradiographed 2 mm sections illustrated discrepancy between vascular volume and perfusion as compared to renal cortex. Tumor areas were heterogeneously supplied with an irregular network of fine-caliber vessels. Histopathological examinations were also undertaken trying to correlate morphology vascular architecture and perfusion.

In summary the cross-sectional area of the precapillary resistance vessels of renal carcinoma is smaller than that of renal cortex. Further the tumor resistance vessel reacts strongly to noradrenaline and is probably hyperreactive compared to renal cortical resistance vessels. The induction of tumor vascular hyperactivity in pharmacological angiography can not be substantiated by this study but the discrepancy is probably due to the vastly differing levels in the vascular arborization being studied.

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ION CONDUCTANCE CHANGES IN HIPPOCAMPAL NEURONES

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When a short current pulse is injected in vitro the membrane potential is changed and this change may be used to calculate the membrane resistance. However the voltage response to hyperpolarising current often deviates from the passive response expected from the membrane. This non-linear response was first described by Ito in the test and later further investigated by Ito and Ohtsuka in cat alpha motoneurons. They suggested that the non-linearities were produced by mechanisms different from the mechanism causing delayed rectification and that more than one ion conductance might be involved.

We have tested if the non-linearities may be due to changes in potassium conductance only by covering recordings from in vitro guinea pig hippocampal slices and the outcome of the simulation of simple computer model of the nerve membrane in the model the potassium conductance (G_K) was time and voltage dependent.

$$\frac{dV}{dt} = \frac{1}{C} \left[G_K (V - E_K) + G_{Na} (V - E_{Na}) + G_{Cl} (V - E_{Cl}) \right]$$

$$G_K = k_1 \exp(k_2(V - V_r))$$

$$G_{Na} = k_3 \exp(k_4(V - V_r))$$

where V_r is the resting membrane potential and the

membrane potential and k_1, k_2, k_3, k_4 chosen constants. Thus the potassium conductance increases during depolarisation and decreases during hyperpolarisation similar to the delayed rectification of the Hodgkin-Huxley model.

The membrane potential was calculated during and after injection of current pulse by solving

$$\frac{dV}{dt} = \frac{1}{C} \left[(I - G_K(V - E_K) - G_{Na}(V - E_{Na}) - G_{Cl}(V - E_{Cl})) \right] / C$$

by numerical recursive method on PDP 11/34 computer. The G are ion conductances and the E are equilibrium potentials. C the membrane capacitance.

The membrane potential calculated in this model after current injections had time courses very close to the time courses recorded from CA1 in the hippocampal slice. The constants k_1, k_2 could be varied over wide range without changing the basic shape of the calculated potentials. It is thus possible that the non-linearities are due to time and voltage dependent change in only potassium conductance. Surprisingly the model also showed anomalous rectification in certain voltage range.

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Cardiac receptors and sympathetic nerve traffic in the spontaneously hypertensive rat (SHR)

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Cardiac mechanoreceptors mainly located in the left atrium in the rat are reset in SHR. The threshold in left atrial pressure (LAP) for activating these receptors is twice as high in SHR compared to the normotensive rat (NR) (Thoren et al 1979). The difference in LAP threshold between SHR and NR for reflex inhibition of the renal sympathetic outflow from left atrial receptors was similar to the threshold difference for the afferent traffic from the receptors (Ricksten et al 1979). To explore whether structural changes of left atrium could explain the resetting in SHR the dynamic distensibility of the nonbeating isolated left atrium was studied in SHR and NR determining relative changes of left atrial volume upon a rapid LAP increase from 2.5 to 12.5 mm Hg. The dynamic distensibility was 25% lower in SHR ($p < 0.01$), which to some extent can explain cardiac reflex resetting. One also has to assume a primary receptor change per se in SHR. In awake rats a blood volume expansion inhibits the splanchnic nerve traffic significantly more in SHR compared to NR (Ricksten et al 1980). This was due to an augmented low pressure receptor response in SHR in spite of the receptor resetting probably due to a decreased distensibility of the capacitance vessels. Therefore LAP was measured in awake SHR and NR during blood volume expansion. From preliminary experiments a 20%

volume load increased LAP significantly more in SHR compared to NR (4.4 vs 2.8) due to a greater centralisation of the volume load because of stiffer capacitance vessels in SHR compared to NR.

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Acta physiol scand Accepted for publication

D 34

Hypothermia and bile production

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It has previously been demonstrated that total bile production is sensitive to small change in body temperature (Krarup & Larsen 1972). The purpose of the present experiments was to examine the effect of temperature on the different fractions of bile production: i.e. canalicular bile acid-dependent and independent bile production and net ductular fluid transport.

The experiments were performed on fasting chloralose-anesthetized cats. Bile was collected from a catheter placed in the choledochus. Bile acids were infused at a rate of $0.2 \mu\text{mol kg}^{-1} \text{ min}^{-1}$. Canalicular bile flow was estimated from the biliary clearance of ^{14}C -erythritol. In the control period body temperature was kept close to 38°C and the temperature was then lowered to 36°C by external cooling.

In 5 experiments cooling caused a significant fall in total bile flow from 13.4 ± 1.8 to $8.9 \pm 1.1 \mu\text{l kg}^{-1} \text{ min}^{-1}$ together with a parallel decrease in canalicular bile flow from 18.6 ± 1.8 to $13.5 \pm 1.3 \mu\text{l kg}^{-1} \text{ min}^{-1}$ without affecting the biliary excretion rate of bile acids and the net ductular fluid absorption. In 5 experiments the net ductular fluid absorption was changed to net fluid secretion by secretin $2 \text{ U kg}^{-1} \text{ h}^{-1}$. Again cooling de-

creased significantly both total bile flow from 22.6 ± 2.3 to $17.5 \pm 1.6 \mu\text{l kg}^{-1} \text{ min}^{-1}$ and canalicular bile production from 19.6 ± 2.7 to $14.8 \pm 2.0 \mu\text{l kg}^{-1} \text{ min}^{-1}$ without affecting the biliary excretion rate of bile acids and the secretin induced net ductular fluid secretion.

Cooling thus decreases the bile acid independent fraction of canalicular bile production apparently without influencing other processes involved in bile production.

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cell aggregation in the renal medulla consti-
tutes an important factor in acute renal failure?

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Acute renal failure was induced by 45 min. clamping
of the renal artery (severe ischemia). A typical pat-
tern of responses appeared with a fall in inter-
stitial blood flow, greatly depressed glomerular
filtration to 5% of the normal, isosthenuria
and an impaired potassium secretion. The reduced
filtration was found to be due to an increased
renal tubular pressure, probably secondary to
tubular obstruction located to the loop of Henle
of the medullary collecting duct.

Although the total blood flow remained largely
unaltered the medullary blood flow was depressed
about 10% of the normal, as investigated with
inhibition extraction technique.

In red cell content of the renal medulla as in-
vestigated from the equilibrium concentration of
51Cr-labelled red cells was about 9.6-10.4% at
normal conditions increasing to 22% at 90 min
ischemia. In conditions of acute renal failure
in the rat red cell seems too to be stagnant
within the vasa recta system.

From these studies the sequence of events providing
the basis for acute renal failure is suggested to be:

1. Primary ischemia evokes functional alterations
of the capillary endothelium with subsequent forma-
tion of red cell aggregates in the vasa recta sys-
tem.

2. These aggregates will then constitute mecha-
nical hindrance to the blood perfusion. The ischemia
will then proceed even after relief of the ischemia.
Electron microscopical examination shows that the ori-
ginal ischemia does not produce any cellular
damage characterized as irreversible (Harvig-79).
The continuing ischemia at revascularization turns
these cells into an irreversible state with intra-
cellular edema and eventually necrosis with re-
jection of cell material into the tubular lumen in
the loop of Henle and the medullary collecting
ducts. The cell debris will have firm attachments
for the urinary passage. As the filtrate moves
through the tubular fluid will be accumulated with
subsequent dilatation of the tubules and increased
hydrostatic pressure up to a level where the filtra-
tion ceases. Microperfusion experiments have verified
these conclusions.

The depressed medullary blood flow will also cause
functional impairment of the renal medulla with
subsequent loss of the urine concentration ability
and the ability to secrete potassium.

It should be pointed out however that the same vas-
cular changes the deposition of red cell aggregates.
The surrounding tubular structures will then remain
intact. These nephrons will be the ones responsible
for the small filtration rate. The number of intact
nephrons will determine whether the renal failure
is polyuric or oliguric but they are on other
principles differences between these two conditions.

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D 36

Correlation between blood level and tissue level of
growth hormone in normal rats

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There are conflicting reports regarding the rela-
tion between bio- and immunoassayable plasma
growth hormone (GH) activity. Ellis and Grim-
mard (1978) have reported that there appears to be
no such correlation in plasma of normal humans
and rats as measured by the rat tibia assay.
This can be accounted for by radioimmunoassay
(RIA). Stewart et al. (1977) found good correla-
tion between bio- and immunoassayable GH activity
in plasma of rats bearing the GH secreting
tumour (PRL171). Both when bio-assayable GH activity
was determined in vitro (uptake of 3-O-methyl
glucose) and in vivo (incorporation of hydraline into
cartilage). The relation between bio-
and immunoassayable GH activity in plasma of
normal animals with an episodic secretory pattern
of GH determined by RIA, has not been studied
in man.

Free behaving 30-day old male rats were decapitated, the blood was collected and plasma levels
of GH were determined by highly specific RIA
(Ijsson et al. 1978). Plasma levels of GH ranging
between 0 and 800 ng/ml were found. Plasma of rats
below (below level lower than 8 ng/ml were pooled
(pool A) and plasma of rats containing more than
80 ng/ml were pooled (pool B). The two separate

plasma pools were diluted with Krebs bicarbonate
buffer (KRB) 1:2. Epididymal adipose tissue of hy-
popophysectomized rats were preincubated with I) plas-
ma from hypophysectomized rat diluted with KRB 1:2
II) KRB (1 g/ml) III) pool A and IV) pool B for
180 min. Subsequently the effect of second addi-
tion of KRB (5 g/ml) in vitro on the production of
CO₂ from radioactive glucose was determined during
the fourth hour of incubation. The second addition
of KRB increased CO₂ production in fat pad preincu-
bated with I) by 45% (SE p 0.05) with II) by 5% (n.s.) with III) by 44% (SE p 0.01) and with IV) by 7% (SE p 0.10).

The plasma of rats with high concentrations of
irradiating GH induced refractoriness to second
exposure of GH. Highly specific response in the
time course of action of GH. The present study sug-
gests good correlation between bio- and immuno-
assayable GH activity in rats.

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The influence of potassium-induced neuronal noradrenaline release on the potassium sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats

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Potassium activates vascular smooth muscle by depolarizing the plasma membrane and thus increasing the permeability of potential controlled Ca channels (Bolton 1979). Recent evidence suggests that the conflicting evidence concerning the K sensitivity of the vasculature from spontaneously hypertensive rats (SHRs) may in part be due to the effects of neuronal noradrenaline (NA) release induced by K stimulation (Yanhouette 1978). We have therefore investigated the K sensitivity of mesenteric resistance vessels (i.d. 150-200 μ m) from young (4-wk BP=52 \pm 3 (SE) mmHg n=10) and adult (4-mo BP 154 \pm 5 mmHg n=3) SHRs. Control vessels were taken from normotensive Wistar Kyoto (WKY) rats (4-wk BP=53 \pm 3 mmHg n 10 4-mo BP=99 \pm 4 mmHg n=9).

Vessel segments were mounted on a myograph (Mulvany and Halpern 1977) and their K-characteristics determined in cumulative K-dose response experiments before and after treatment to eliminate the effect of K induced neuronal noradrenaline release. Treatment consisted of destroying the nerve terminals in vitro with 6-hydroxydopamine (Apprigliano & Hermsmeider 1976) or of blocking the α -receptors with phentolamine (1 μ M).

The treatment (a) reduced the maximum K response (with 125 mM K⁺) substantially but (b) did not affect the K responses at K-concentrations below

about 50 mM. Thus the treatment did not affect threshold K sensitivity but reduced the K-ED₅₀ concentrations. The reduction in the maximum K-responses caused by treatment was similar in SHR and vessels but greater in adult vessels (53 \pm 21) than in the young vessels (36 \pm 35). Before treatment adult SHR vessels were more ($P < 0.01$) sensitive (K ED₅₀=48 \pm 20 mM) than the WKY vessels (K ED₅₀=46 mM) but there was no difference in the K sensitivities of the young vessels (K ED₅₀=66 \pm 3 mM (SHR) 63 \pm 3 mM (WKY)). After treatment there was no difference in the sensitivities of the adult W (K ED₅₀ 39 \pm 1 mM (SHR) 37 \pm 1 mM (WKY)), but the j SHR vessels were less ($P < 0.1$) sensitive (K ED₅₀ 44 \pm 1 mM).

The results therefore suggest that K induced neuronal NA release has a large influence on the K responses of mesenteric resistance vessels. The increased K sensitivity of untreated adult vessels does not seem to be due to an increased sensitivity of the smooth muscle within these vessels but rather to the decreased K sensitivity of the young treated SHR vessels suggests because it precedes development of elevated blood pressure that an initially decreased K sensitivity in the vessel could be a factor which is involved in the etiology of hypertension in the SHR.

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D 38

Facilitatory interaction between reflex pathways from muscle afferent of different diameter

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A wide convergence of different afferent onto common interneurons in reflex pathways from group I and II muscle afferents to α -motoneurons has been shown by testing specific facilitation (Pedina & Hultborn 1972; Lundberg, Malmgren & Schomburg 1977, 1978). The technique of selective chemical ablation of group III and IV muscle afferent (Hense 1977) enabled the investigation of their influence upon other reflex pathways without contaminating effects from low threshold afferent.

In high spinal cat motoneurons 2 hindlimb flexors (Pso⁺ Par⁺) and extensors (Abdo⁺ GS Tib⁺) were intracellularly recorded and the effect of group III and IV muscle afferent activated by intraarterial injection of potassium chloride or bradykinin into the GS muscle upon reflex responses evoked by electrical stimulation of group I and II afferent from different muscle groups was tested. The reflex responses were elicited to the different afferent fibre groups according to their threshold stimulus strength.

EPSP evoked by group Ib II or III muscle afferents were almost generally facilitated by the afferent inflow from the chemically isolated group III and IV muscle afferents. Therefore it was irrelevant from which muscle the group I III afferent were originating and if they projected onto a flexor or extensor motoneurons except that Ib EPSPs in extensor motoneurons were not observed. The facilitation often outlasted the synaptic membrane effects evoked by the group III and IV afferents (Kniffki, Schomburg & Steffens 1978). This fact together with the observation that monosynaptic EPSP were not enhanced suggests that the facilitation are not due to changes of the motoneuron membrane. Group Ib and II EPSPs principally could be facilitated but the result were more variable and here often an influence of the changed motoneuron membrane potential could not be excluded.

The results indicate that there is an extensor convergence from group III and IV muscle afferents onto common interneurons in reflex pathways from group Ib II and III muscle afferent. Since receptors of group III and IV muscle afferents are not only nociceptive but also can be activated muscle stretch and contraction (Kniffki, Hense, Schmidt 1978) it appears that the afferent of different muscle receptors may co-operate in the control of normal movements.

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relationship between insulin and insulin binding and response to insulin in isolated fat cells from normal, ventromedial hypothalamus lesioned and diabetic rats

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Insulin binding and ^{14}C labelled glucose incorporation into fatty acids were studied in isolated fat cells from ventromedial hypothalamus (VMH) lesioned rats (Mustvedt & Nielsen 1972) weighing more than 350 g in pair matched controls and in rats weighing about 240 g with or without streptozotocin-induced diabetes.

The insulin binding was significantly increased in adipocytes from VMH lesioned (P < 0.01) and diabetic (P < 0.02) rats compared to control rats (Table I). The increased binding in diabetic rats was associated with lower plasma insulin concentration. In contrast, the insulin level in VMH lesioned rats tended to be elevated indicating that factors other than plasma insulin regulate the receptor concentration.

The glucose incorporation into fatty acids was considerably elevated in the VMH lesioned rats as compared with weight matched controls (P < 0.01). Streptozotocin-in-

duced insulin depletion lowered the glucose incorporation into fatty acids (P < 0.01, Table I) even though receptor binding was increased. The results suggest that the reduction of insulin-induced fatty acid synthesis in VMH lesioned rats is not caused by the increased binding to the receptors.

Table I. Insulin binding and insulin-stimulated fatty acid synthesis.

Isolated adipocytes were incubated at 37°C for 90 min with 25 pM [^{125}I]-moniodoinsulin (Nilsson & Somme 1978). Results are expressed as specific binding (total binding minus binding in presence of 1 pM unlabelled insulin) as cpm per cell/cpm per 1 medium 10^{-11} i.e. fractional binding. Physiological concentration. Fatty acid synthesis. Isolated adipocytes were incubated in the presence of maximal concentration of insulin and 4.7 mM ($U-^{14}C$) glucose for 2 hours at 37°C. The lipids were extracted, hydrolysed and the ^{14}C -activity was measured in the fatty acids. Results are expressed as nmol glucose converted/ 10^5 cells/2 hours. Median and range.

| Group | 240 g n=10 | Diabetic n=10 | VMH n=12 | 350 g n=7 |
|-------------|---------------|------------------|-------------|--------------|
| Binding | 1.5 | 2.6 | 2.9 | 1.6 |
| Insulin | 0.8-1.9 | 0.7-3.4 | 1.7-4.0 | 1.0-2.7 |
| Fatty acids | 10 | 1 | 11 | 9 |
| | 5-99 | 0-5 | 10-229 | 0-20 |

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D 40

Effect of 5-hydroxytryptamine on the release of intestinal polypeptides in the presence of cholera toxin

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It was shown from this laboratory that intracerebral nerves markedly stimulate the intestinal secretion induced by cholera enterotoxin (Casato et al. 1979). In these results and on previous findings from other laboratories the effect of leaving hypothalamus was proposed for the nervous mediated release of secretin as a reflex arc (Mustvedt & Nielsen 1972) via the release of 5-hydroxytryptamine (5-HT) from the cat mesencephalon (EC) cells. The 5-HT stimulates and is located mainly adjacent to the EC cells. One of the secretors released is the secretin and the effect of the secretin is the release of the intestinal polypeptide. The following experiment was performed to test this hypothesis. Rats exhibiting cholera secretion were either treated with atropine against secretin or 5-HT receptor blocking agent (metysergide, metysergine) or given intravenously. All these experimental manipulations inhibited the cholera secretion and in most cases an increase in secretion was observed. The total effect was decreased secretion of about 60% in the total trans-

port change caused by the cholera toxin. In accordance with these results histochemical observations the EC cells in cats show marked reduction of 5-HT content after the action of cholera toxin (Nilsson et al. this supplement). In cat experiment the release of VIP into the blood from the gut was determined in control animal and in animals exposed to cholera toxin. During 5 h of incubation period there was no timeous decrease in the VIP release in the control animal while the VIP release increased steadily from intestine exposed to the cholera toxin. This release could be inhibited by tetraol toxin given intravenously or lidocaine administered into the gut lumen. The present experiments are thus in full agreement with the hypothesis outlined above.

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The effect of high fat diet on adipose tissue lipoprotein lipase activity in rat

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Human obesity can be divided into two main categories. Usually childhood obesity is hyperplastic while the adult obesity is hyperplastic. Hyperplastic obesity can be studied with Zucker rats which are genetically obese (Gruen & L. 1978). The best approach for hyperplastic type is probably through dietary manipulations.

The lipoprotein lipase (LPL) enzyme catalyzes hydrolysis of circulating triglycerides releasing free fatty acids to be transported to adipocytes. Thus this enzyme is of importance in the development of obesity (Gruen & L. 1978; Nietanen & Greenwood 1977). In the present study rats were fed high fat diets and the effect of these diets on the adipose tissue LPL activity were studied.

Rats were fed high fat diet (24.5 J/g) high fat diet supplemented with extra protein (23.9 J/g). Dietary protein content was 18.2% in normal high fat diet and 36.5% in protein-enriched high fat diet. The feeding was continued at weaning and if first control had only high fat diet while after 3 weeks rats were divided to two groups having both high fat diet but different protein supplementation. Feeding these experimental diets was for the continued 6 weeks. Rats were killed and epididymal fat pads were removed. The fat pads were homogenized in 0.25 M sucrose-1 mM EDTA and postmitochondrial supernatant was prepared for enzyme

assays. The lipoprotein lipase (LPL) was determined as described by Nietanen & Greenwood (1978). The adipose tissue DNA content (Greenwood & Nietanen 1978) and protein content of the postmitochondrial supernatant (Lowry & L. 1951) were measured.

The weight gain was significantly faster in rats having high fat diet supplemented with protein in 7 pad weight and relative fat pad weights did not differ between groups but if compared to rats fed diet with normal fat content the relative fat pad weights were higher in those rats fed high fat diet. The DNA content was significantly higher in those fat pads taken from rats having high fat diet than in those having high fat diet supplemented with protein. The LPL activity was significantly higher in the high fat group than in the high fat group with protein supplement when expressed per wet weight (13.2 vs 5.4 U/mg of FFA/h). Also protein basis the LPL activity was higher in the high fat group without protein supplementation. However when the activity is expressed per mg of DNA there was no significant difference in the activity.

The results suggest that in rats having high fat diet the LPL activity increases precedes the enlargement of adipocytes and coincide with the rat proliferation. Grant: Y. Jahnson Foundation (Finland).

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D 42

Regulation of glycogen phosphorylase and glycogen synthetase during exercise in man

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This study was undertaken to determine if the increased rate of glycogen breakdown during exercise was due to an increase in phosphorylase b activity and to investigate the effect of

AMP in this process. Seven subjects worked to exhaustion on an ergometer cycle (4.8 min). Muscle samples were taken by the needle biopsy technique before and after exercise and were analysed for cAMP, glycogen phosphorylase and glycogen synthetase I and D, lactate and hexo-phosphates. The muscle content of cAMP increased about 100% during exercise and the active form of glycogen synthetase decreased from about 40% of the total activity to about 10% after exercise. An unexpected finding, however, was that phosphorylase decreased from about 10% of the total activity to about 4% after exercise. Blockade of the receptors before exercise with propranolol inhibited the increase in cAMP and the glycogen synthetase translocation during exercise. Phosphorylase was affected less with -blockade further decreased (about 1% of the total phosphorylase activity). Accumulation of lactate and glycogen 6-phosphate in muscle were

enhanced by exercise with -blockade (84 and 2.2 mmol/g dry wt respectively) compared to exercise without blockade (101 and 8.7 mmol/g dry wt respectively).

inactions in vacuola permeability [induced by
free radical]

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oxygen toxicity is suggested to be mediated by
production of reactive intermediates as during
biological reduction of oxygen to water. Al-
though most oxygen is reduced directly to water
in the addition of four electrons by cytochrome
c, some univalent reduction occurs resulting in
peroxide radical (O_2^-), hydrogen peroxide (H_2O_2)
and hydroxyl radical (OH^\cdot). Many oxidative pro-
cesses of biological importance have been shown
to produce O_2^- in vitro. Experiment illustrates
the damaging effects of O_2^- appears to depend
on the simultaneous presence of H_2O_2 which can
be formed by the spontaneous dismutation of O_2^- .
Reason for this seems to be the O_2^- and H_2O_2
react to form OH^\cdot which is one of the most
active oxidizing species known.

Physiologic defense mechanisms against these reac-
tive oxygen intermediates have evolved: superoxi-
dismutase (SOD), catalase (CAT) and gluta-
thione peroxidase. In addition to a-tocopherol
and ubiquinol. These protective mechanisms are
only intracellular leaving the extracellular
space more susceptible to oxygen radical attack.
Polymorphonuclear leukocytes which participate in
inflammatory reactions can be activated by a
variety of stimuli to greatly increase their oxygen
consumption. This "respiratory burst" is associ-
ated with the production of O_2^- , some of which is

leaked into the extracellular space where it may
give rise to more reactive oxidizing species such
as OH^\cdot .

We have studied the effect of enzymatically pro-
duced oxygen derived free radical on cellular per-
meability using the hamster cheek pouch model.
FITC-dextran (MW 150,000) was injected intravascularly
as permeability tracer and O_2^- and H_2O_2 generation
accomplished by the topical application of xan-
thine oxidase and hypoxanthine. This caused a
significant increase in leakage per cm. The
simultaneous application of either SOD, CAT or
methyl sulfoxide or L-methionine significantly re-
duced the leakage suggesting OH^\cdot the main da-
maging species.

These results suggest that oxygen derived free ra-
dical may cause an increase in vascular permea-
bility and the degree of the increase in permea-
bility seen in inflammation might be related to
oxygen radicals produced by the activity of invading
leukocytes.

The effect of high fat diet on adipose tissue lipoprotein lipase activity in rat

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D 42

Regulation of glycogen phosphorylase and glycogen synthetase during exercise in man

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This study was undertaken to determine if the increased rate of glycogen breakdown during exercise was due to an activation of phosphorylase b and to investigate the role of

AMP in this process. Seven subjects worked to exhaustion on an ergometer cycle (4-6 min). Muscle samples were taken by the needle biopsy technique before and after exercise and were analysed for cAMP, glycogen phosphorylase and glycogen synthetase I and II. The endogenous phosphatase. The muscle content of cAMP increased about 100% during exercise and the active form of glycogen synthetase decreased from about 40% of the total activity to about 20% after exercise. An unexpected finding was however that phosphorylase decreased from about 10% of the total activity to about 4% after exercise. Blockade of the receptors before exercise with propranolol inhibited the increase in cAMP and the glycogen synthetase translocation during exercise. Phosphorylase a was affected little with blockade further decreased (about 1% of the total phosphorylase activity). Accumulation of lactate and glycogen 6-phosphatase in muscle were

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